

Analysis of Infectious Diseases in

Conditional Mouse Mutants

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Abbreviations

BAC	Bacterial artificial chromosome
Cre	Causes recombination of the bacteriophage P1
DC	Dendritic cell
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
ES	Embryonic stem
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FRT	Flp recognition target
M-CSF	Macrophage colony-stimulating factor
MnSOD	Manganese Superoxide Dismutase
H& E	Hematoxylin & eosin
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
<i>Lm</i>	<i>Lysteria monocytogenes</i>
loxP	Locus of crossing over (x) P1
Lys	Lysozyme
JAK	Janus Kinase
neo	Neomycin
NK	Natural killer
ROS	Reactive oxygen species
RT	Room temperature
SCID	Severe combined immunodeficiency
STAT	Signal transducer and activator of transcription
TH	T-helper

1. Introduction

1.1 General Introduction

The immune system consists of specialized cells that settle in the periphery of the body or in the lymphoid organs. They are capable of discriminating between self versus non-self organisms. The cells that form the immune system are of haematopoietic origin. The respective precursor cells can be found in the bone marrow in adult mammals. The immune system is divided into an innate and an adaptive component [1]. Within both of these systems, there are specialized cells that communicate via direct interaction of surface molecules and through the distribution of messengers (e.g., cytokines) [2-4]. “Cytokine” is a collective term for peptide molecules with regulatory effects secreted by cells. Thus, this term comprises a heterogeneous group of molecules [5].

The innate immune system is characterized by the rapidity in the response to danger signals. Innate mechanism of self defense were acquired by vertebrates long before the evolution of an adaptive immune system. Even single-cell organisms have heritable defense mechanisms, and every multicellular organism appears to have a complex innate immune system [6]. The basic protective strategy of an innate immune system is for the organism to constitutively produce generic receptors that recognize conserved patterns on different classes of pathogens to trigger an inflammatory response that limits pathogen invasion [7-9]. By contrast, specific adaptive immunity depends upon the somatic diversification of antigen-receptor genes to generate vast repertoire of cells, each of which expresses a different antigen receptor. Lymphocytes, the specialized cell type of adaptive immunity, use their cell-surface receptors to recognize antigenic configuration of specific pathogens. The response to antigen triggering consists of clonal amplification, cellular differentiation, and production of antibodies with the same antigen binding specificity [10]. The adaptive immune response is usually more effective, but it requires a certain amount of time to be initiated. However, T- and B-cells can differentiate

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into memory cells that enable a faster specific immune answer in case of repeated contact with the pathogen.

Among the most important cells in the innate immune system are phagocytic cells such as neutrophils, granulocytes, and macrophages [7]. These cells identify pathogenic agents by means of rather general and recurrent structures, for example on their surface (so-called PAMPS = pathogen associated molecular patterns) [9]. They then phagocytose the pathogens and secrete messengers, which in turn attract and activate further immune cells. This part of the immune system can be activated rapidly and thereby forms a primary defense line.

The bactericidal action is made possible by several means, such as the production of reactive oxygen species (ROS) [11]. The synthesis of ROS is a physiological event, as several metabolic processes lead to the release of reactive intermediates as by-products of their reactions [12]. Besides being extremely harmful for the pathogen, these reactive molecules are also dangerous for the host cells. This is the reason why numerous enzymes are involved in their detoxification [13].

Signal molecules play a crucial part in the regulation of the immune defense. Cytokines and chemokines are synthesized and secreted by many cell types upon contact with a pathogen. They can increase cell activity autocrinously in a positive feedback, or they can activate surrounding cells paracrinely. Chemokines mainly attract further immune cells, whereas cytokines have a number of rather diverse effects. Among other functions, they can stimulate or inhibit cell proliferation, or act as differentiating factors for certain cell types [14].

Like all signal molecules, they bind to specific receptors on the target cell. This in turn activates a signal cascade that will cause a change in transcriptional activity of the cell. The gene transcription is regulated by the interaction of transcription factors, co-factors, specific binding sequences on the DNA, and the conformation of the DNA [15-17]. Not only the signals mediated by the cytokines, but also the signals mediated by the specific T- and B-cell receptors play an important part in the immune system. During development, the signals

mediated by these receptors are aimed at selecting cells with appropriate receptors [18]. Later on, the binding of the relevant antigen to the receptor, along with other co-factors such as cytokines, activates T- and B-cells and also in this process, the signals are translated into a change in gene regulation within the cell, thus they affect the activity of various transcription factors [19].

1.2 The mouse as a model organism

Research on the different components of the immune system and their regulation, as well as the mechanisms leading to a collapse of the immune system, would be hard to imagine without the mouse as a model organism.

Sequencing of murine and human genome revealed that 95% of the coding sequence is conserved at the DNA level [20, 21]. Likewise, the immune system of the mouse shows a considerable similarity to the human immune system. Many human diseases can be found as well as induced in mice. Hence, at least part of the data derived from mice generalizes to immunological processes in humans. Moreover, mice are relatively easy to breed and keep.

Various in-bred lines ensure reproducibility of the results because these mice (like clones) do not exhibit any genetic differences. The greatest advantage, however, concerns the possibility to induce systematic changes in the mouse genome and to examine their effects *in vivo* [22].

When both alleles of a gene have been deactivated completely by a certain mutation, a knock-out mouse has been bred. With such mouse mutants, it is possible to examine the phenotypic effects of a specific gene loss.

Conditional mutants have proven of value in cases where the complete loss of a gene leads to a lethal defect, or to address special research questions [23, 24]. In these animals, the target gene or parts of it (e.g., an exon) is flanked in both alleles by two identification sequences for Cre recombinase (loxP sequences; lox = locus of crossing over). This is also called floxed

allele. By inserting the loxP sequences in non-coding parts of the gene, the latter remains intact and does not cause a phenotype. Only the expression of Cre recombinase within the same cell leads to the loss of the DNA section located between the loxP sequences, and thus to the inactivation of the respective gene.

There are several mouse lines in which Cre is expressed under control of cell type-specific or inducible promoters, with either ubiquitous or tissue-specific induction. The advantage of conditional mutagenesis concerns the possibility to systematically knock out genes in specific cell types and study the effects. In the context of immune system research, this approach offers the opportunity to identify the biologically relevant cells for certain processes. Examples include infection and autoimmunity research.

1.3 Signal transduction in the immune system: Interleukin 12 (IL-12) and its cytokine family

Interleukin is a historical term referring to signal molecules that serve the communication between leukocytes. This narrow definition is no longer valid because various interleukins also transfer signals to cells that do not belong to the haematopoietic system. Cytokines and their specific receptors are classified into different families according to structural similarities. The cytokine families are: haematopoietics, interferons, chemokines, and tumor necrosis factors. The cytokine receptors are distributed among the cytokine receptor family-1 or haematopoietin receptor-superfamily, cytokine receptor family-2 or interferon receptor-superfamily, and the tumor necrosis factor receptors.

Cytokines and chemokines form the basis for communication between cells involved in the immune defense. As described above, the immune system can be divided into the innate or unspecific immune defense and the adaptive or specific immune defense. For both parts, there are specialized cells as well as specialized cytokines that are characteristic of the immune

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reaction. IL-12 belongs to the type 1 or pro-inflammatory cytokines that are characteristic of the innate immunity and promote the initiation of an inflammatory reaction [25].

IL-12 is mainly produced by phagocytic cells such as macrophages and neutrophils, representing the first defense line against invading pathogens, as well as dendritic cells (DC), whose role is to present antigens and activate T-cells.

The invasion of a pathogenic agent is primarily identified by the innate immune system. This involves phagocytic cells such as granulocytes and macrophages and the antigen-presenting dendritic cells. These cells then release IL-12 as one of the first cytokines [26, 27]. IL-12 is the first identified heterodimeric cytokine. It consists of two subunits p35 and p40 that are covalently bound.

The DNA sequences of both subunits are located on different chromosomes both in mice and humans, and their expression is regulated independently of one another [28].

The murine gene for p35 is located on chromosome 3 and contains 7 exons. The p40 gene is located on chromosome 11 and consists of 8 exons whose length is approximately 14 kb [27, 29]. IL-12 is secreted only when both genes are transcribed in a cell. While the expression of p35 has been verified in a large variety of cell types, the expression of p40 is restricted to those cell types that can also secrete IL-12 [30, 31]. As opposed to the p35 subunit, the p40 subunit can be secreted alone and forms homodimers in mice. These can interact with the receptor and thereby act as a competitive inhibitor [32, 33]. However, the biological function of homodimers is controversial [34-36].

Sequence analyses yielded a close relationship between IL-12 and interleukin-6 (IL-6). IL-6 binds to its receptor as a homodimer, consisting of the IL-6R α chain and a gp130 chain. The gp130 chain serves as a signal transduction chain for various interleukin receptors. Moreover, there is a soluble form of IL-6R α that can also bind IL-6 and can associate with membrane-bound gp130. In this manner, even those cells that do not express the IL6R α chain can become susceptible to IL-6.

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IL-12 p35 shows a considerable homology to IL-6, whereas p40 is homologous to the IL-6R α receptor chain. Both of the IL-12 receptor chains, IL-12R1 and IL12R2, are in turn homologous to gp130. This similarity prompts the conclusion that IL-12 has derived from an early form of IL-6 and its soluble receptor [37-39].

The target cells of IL-12 are natural killer cells (NK-cells) and T-cells. The latter express the IL-12 receptor which comprises two subunits. Because of their homology to β -type cytokine receptors, these subunits are classified as IL-12R β 1 and IL-12R β 2. In addition to GP130, this group of receptors includes the leukemia-inhibitory factor receptor (LIF receptor) [40].

In mice, the IL-12R β 1 chain is mainly responsible for binding the ligand, while the β 2 chain is involved in signal transduction [41, 42]. The binding of IL-12 to its receptor leads to an activation of the JAK-STAT signal cascade (JANus Kinase and Signal Transducer and Activator of Transcription). Transphosphorylation of the receptor-associated kinases JAK2 and Tyk2 leads to a prevailing binding of STAT4 molecules, which in turn are phosphorylated. In the nucleus, STAT4 homodimers can stimulate the transcription of target genes such as IFN γ [43]. Moreover, IL-12 is involved in the differentiation and proliferation of Th1-cells [44, 45], the maturation of cytotoxic T-cells [46], and the proliferation and activation of NK-cells [47, 48].

Even though IL-12 was originally discovered because of its stimulating effect on the cytotoxicity of NK-cells [26], the most important function of IL-12 concerns the activation of the IFN γ expression by T-cells and NK-cells [49]. Such an expression can induce and polarize the T-helper response towards the cell-mediated immune answer (Th1). The Th1 response refers to the reaction of the organism to primarily intra-cellular pathogens (e.g., mycobacteria) in which naïve CD4⁺ Th0 cells increasingly develop into TH1 cells, while at the same time inhibiting the development of Th2 cells. Th1 and Th2 cells mainly differ in the kinds of cytokines they produce. TH1 cells predominantly secrete IFN γ , Interleukin 2 (IL-2), and

tumor necrosis factor- β (TNF β), also termed “lymphotoxin” [50]. These cytokines have an activating effect on macrophages, NK-cells, and neutrophils, i.e. on the cellular components of the innate immunity. Moreover, the Th1 immune response is characterized by an activation of cytotoxic (CD8⁺) T-cells, as well as an expression of isotype IgG2a antibodies that can interact with the complement system. Recently, the Th1/Th2 paradigm has been revolutionized by the discovery of new cytokines in the IL-12 family and of the utterly new IL-17 family.

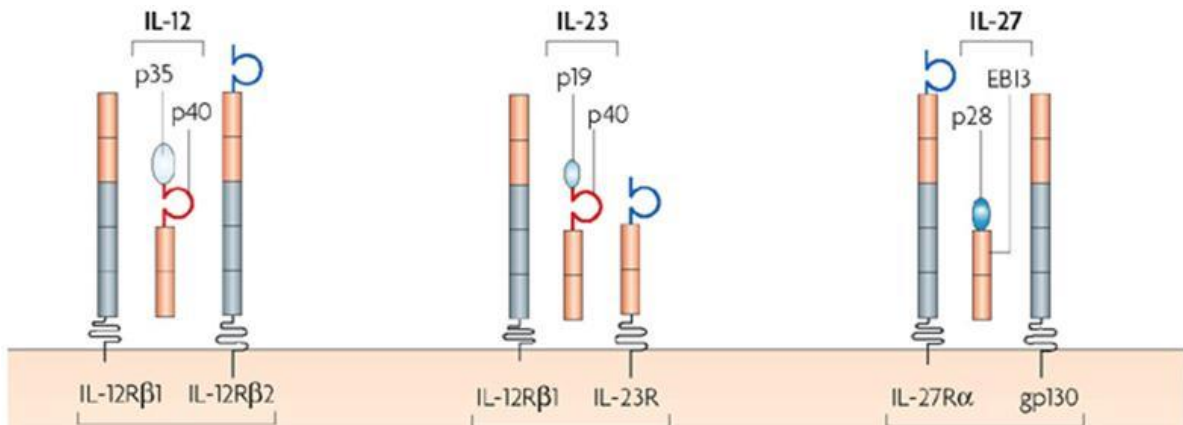


Figure 1: IL-12 family of cytokines. Members of the interleukin-12 (IL-12) family of cytokines comprise a helical subunit (blue ovals) and a receptor-like β -chain. IL-12p70 and IL-23 share a common chain (p40) and their receptors share the IL-12 receptor β 1 (IL-12R β 1) chain. Obtained from [51].

Th17 cells were proven to be the principal pathogenic effectors in several types of autoimmunity previously thought to be Th1-mediated. In 2000 a new member of the IL-12 family was discovered, IL-23. IL-23 is composed of the p40 subunit (the same subunit of IL-12), and the p19 subunit [52] (cf. Figure 1). The receptor consists of the IL-12R β 1 chain and the IL-23R chain [53]. IL-23 shares part of its functions with IL-12, but there are several important differences. IL-23 is secreted mainly by activated DCs. As opposed to IL-12, it cannot stimulate the proliferation of naïve CD4⁺ T-cells. Moreover mice deficient in IL-23 lose development of a population of an IL-17-expressing subset of CD4 T cells [54]. While IL-12 is the master regulator of the Th1 immune response, IL-23 is a key element in the

differentiation of Th0 cells to Th17, and this kind of immunity is particularly important for host protection against extracellular bacteria and some fungi.

1.4 Phenotype of conventional knock-out mice for the IL-12 subunits p40 (p40^{-/-}) and p35 (p35^{-/-})

For both of the IL-12 subunits, knock-out mice were generated and analyzed [55-57]. The mice of both lines developed normally and showed no changes in organ development, haematopoiesis, or fertility. However, mice with mutations in the IL-12 gene are susceptible to intracellular pathogens such as *Listeria monocytogenes*(*add ref*), *Taxoplasma gondii* [58], *Leishmania major* [59], and various mycobacteria, including normally apathogenic strains [60].

IL-12 has been shown to play an essential part in the production of IFN γ in reaction to intruding pathogens. However a small fraction of IFN γ is produced independently of IL-12 [55, 57]. The Th1 immune response of IL-12 p40^{-/-} mice could not be restored completely by a treatment with recombinant IL-12 (rIL-12), which proves that a Th1 immune response requires both IFN γ and IL-12 [55]. Contrary to expectations, differences between p35^{-/-} mice and p40^{-/-} mice have been found in a number of infection experiments. The p35^{-/-} mice tend to be less vulnerable to pathogens or show a less severe etiopathology. These findings gave rise to speculations whether the p40 homodimers that can be formed in p35^{-/-} mice play a role in this context [61]. However, the discovery of IL-23 showed that the inactivation of p40 results in knocking out two interleukins at the same time. Since IL-12 and IL-23 share some functions, the differences between p35^{-/-} mice and p40^{-/-} mice are probably due to a lack of IL-23 in the latter, rather than the lack of p40 homodimers [62]. In any case, the biological relevance of the homodimers remains controversial and needs to be taken into account in a later analysis of mice with the conditional knock-out of the p40 gene.

1.5 Manganese Superoxide Dismutase

Manganese Superoxide Dismutase (MnSOD, SOD2) is a mitochondrial antioxidant enzyme belonging to the SOD gene family. Its main function is to catalyze the dismutation of the superoxide anion into molecular oxygen and hydrogen peroxide [63]. The importance of this gene family is demonstrated by their presence in nearly all organisms. There are several forms of this protein in mammals: SOD1 (Cu-ZnSOD), SOD2 (MnSOD), and SOD3 (extracellular EC-SOD). SOD1 is abundant in the cytosol and in the intermembrane space of mitochondria [64]. In mitochondria, SOD1 has recently been shown to cooperate with cytochrome-c to trigger apoptosis upon leaking out into the cytosol [65]. SOD3 is an extracellular form and is constitutively expressed.

MnSOD is a homotetramer containing one manganese atom in each subunit. Its gene is composed of 5 exons. Both the dimerization domain and the manganese-binding site are contained in the third exon. Inside the cell, SOD2 is located in the mitochondrial matrix, where it controls the cellular redox environment and regulates superoxide and hydrogen peroxide signaling [66]. Its distribution in the organs is directly proportional to energy consumption rates, thus it is expressed mainly in heart, brain, liver, and kidneys [67].

In physiological conditions, the major role of MnSOD is to protect against the reactive oxygen species (ROS) generated in the mitochondria as a byproduct of electron transport and oxidative phosphorylation. In the absence of SOD2, there will be an accumulation of superoxide anions inside the mitochondria. This can impair mitochondrial functions by directly reacting with molecular targets or combining with nitric oxide and in turn be transformed in the highly reactive peroxinitrite or hydroxyl radical. SOD2 expression can be induced both by chemical stimuli (cytokines like IL-1 β , TNF, IL-4, TNF- α and LPS) and by physical ones [68-72].

Knock-outs of Sod1 and Sod3 have been generated and are viable. In contrast, Sod2 knock-outs display a severe phenotype, with effects ranging from embryonic dilated cardiomyopathy and in utero death [73] to early post-natal death (day five after birth) due to hepatic steatosis, severe mitochondrial defects, and massive dilated cardiomyopathy [74, 75]

Depending on the genetic background, the inactivation of the gene has been reported to have various effects ranging from neurodegeneration to cardiomyopathy, but in any case leading to death within 18 days after birth [76, 77].

The SOD2^{+/-} mouse model has been widely used in pharmacological and toxicological studies as well as research on aging, cardiovascular diseases, and neurodegenerative disorders [78-82]. However, to date no direct involvement in infectious diseases has been investigated because of the considerably short life-span of the available murine models. To circumvent this problem, we used the Cre-loxP system to generate a conditional knock-out mouse line, in which the gene inactivation takes place only in macrophages and neutrophils [83]. The first line of defense against invading pathogens is represented by phagocytes of the innate immune system. Subsequent to phagocytosis, the NADPH oxidase complex is assembled [84], and a massive amount of ROS is produced which is critical for the bactericidal action [85]. A hallmark of this event is the transient abnormal uptake of oxygen, the so-called respiratory burst.

SOD2 is localized in the mitochondria, an organelle whose high sensitivity to oxidative stress and key role in intrinsic apoptosis pathway have been demonstrated widely [86-88]. Moreover, it has been observed that SOD2 is up-regulated following infection with Group A *Streptococcus* [89]. These findings suggest that SOD2 is involved in detoxifying the highly dangerous oxygen radicals produced to challenge the infection.

1.6 *Streptococcus pyogenes*

Streptococcus pyogenes is an extracellular Gram+ pathogen responsible for a broad spectrum of diseases, including mild infections of the skin and the upper respiratory tract, bacteremia, and in most severe cases necrotizing fasciitis, sepsis, and septic shock.[90, 91]. It is classified as Group A *Streptococcus* (GAS) because of the induction of β -hemolysis on blood agar. Virulence factors such as lipoteichoic acid enable bacterial invasion of epithelial tissues [92]. The hyaluronic acid capsule has been shown to prevent phagocytosis [93], while M-protein confers resistance to phagocytosis by inhibiting activation of the alternative complement pathway [94]. Resident macrophages play a key role in controlling GAS infections[95, 96]. They recognize, phagocyte, and destroy bacteria. Moreover, they produce cytokines and chemokines that are crucial in controlling the recruitment and activation of inflammatory cells, mainly PMNs, at the site of infection [95, 96]. It is generally assumed that the activation of macrophages is directed toward the elimination of the invading pathogen. However it can also be the case that an excessive and unregulated stimulation may lead to a continuous release of pro-inflammatory mediators, whose synergy can possibly result in septic shock [97]. In the present study, the KTL-3 strain of *Streptococcus pyogenes* was employed in subcutaneous infection models. The GAS KTL-3 strain, also referred to as flesh-eating bacteria, was first isolated from the blood of a patient with streptococcal bacteremia [98]. The ability of such bacterial strain to escape host defenses and invading soft tissues raised interest about these particularly aggressive bacteria. In susceptible mouse strains, bacteria injected subcutaneously were found in the blood three days after infection, and these mice eventually died of septic shock [99]. *S. pyogenes*-induced macrophage activation results in the up-regulation of several pro-inflammatory and bactericidal factors[89]. Interestingly, MnSOD gene transcription levels were four-fold higher than in uninfected macrophages. Hence, investigating the role of MnSOD in streptococcal infections may shed light on this unexpected phenotype observed during *in-vitro* experiments.

1.7 *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is an extracellular Gram+ pathogen capable of causing a diverse spectrum of diseases such as cutaneous and invasive infections [100]. It can colonize the skin and mucosal surfaces in an asymptomatic manner. Moreover, in severe invasive infections, it can lead to sepsis, pneumonia, and endocarditis [101]. The emergence of the multi-drug resistant *S. aureus* has become a major public health concern [102, 103]. While the reason for susceptibility to *S. aureus* remains unknown, there is increasing evidence that aside from bacterial pathogenicity factors, host genetic factors might play a critical role in determining susceptibility to *S. aureus* infections [104]. The pathogenicity of *S. aureus* is due to the ability of producing a repertoire of toxins, exoenzymes, adhesins, and immune-modulating proteins [105, 106]. No single virulence factor has been proven to be sufficient to provoke a staphylococcal infection. Such infection is rather promoted by the coordinated action of various virulence factors, the expression of which is controlled by several regulatory systems [107-109]. Staphylococcal sepsis often begins from infected loci at the body surface such as skin and soft tissue infections, and induces an influx of neutrophils [110]. Tissue invasion and killing of phagocytes are involved in the inflammatory response that leads to septic shock [111]. In this model of infection, the kidney has been shown to be the main target organ [112]. Genetic control of immunity to *S. aureus* remains poorly understood, mostly because of the complex host-pathogen interactions that determine the outcome of infection. In this study, the intravenous infection model was used to evaluate MnSOD role as a potential host resistance factor.

1.8 *Listeria monocytogenes*

Listeria monocytogenes (*L. monocytogenes*, *Lm*) is a Gram+ intracellular bacterial pathogen causing disease in immunocompromised individuals and pregnant women [113]. Listeriosis

typically occurs after ingestion of *L. monocytogenes* in contaminated food, such as pre-packaged meat and cheese [114, 115]. Infections by contaminated foods can cause gastroenteritis, meningitis, meningoencephalitis, and abortion [113]. Ingested *Listeria* reaches the large intestine, where InlA promotes listerial uptake into intestinal epithelial cells by targeting the adhesion molecule E-cadherin (hEC1) [116]. In case of systemic infection, bacteria enter the circulation and mainly target liver and spleen, where they infect hepatocytes and macrophages [117, 118]. Secondary infections are made possible by the second listerial invasion protein InlB which recognizes the HGF receptor, thus allowing access to several cell types [119-121].

The lack of InlA-HGF interaction in mice is a major difference with humans, that led to the development of the intravenous infection model. This mouse model provides a good system to study innate and adaptive immune responses. Bacteria are internalized by the host cell into a phagosome, from which they escape by means of the expression of virulence factors. The bacteria further replicate into the cytosol and spread to neighboring cells without lysing the primary host cell. This intracellular life-cycle allows *Listeria* both to evade humoral defenses in the extracellular compartment and to escape the harsh environment of the phagosome.

Both innate and adaptive immune responses are responsible for challenging *L. monocytogenes* infection [115]. Following infection with *Lm*, innate immune responses are rapidly triggered and are essential for host survival [122]. The effectiveness of innate immunity to *L. monocytogenes* was shown by studies using Severe Combined Immunodeficient (SCID) mice and nude mice (mice lacking the thymus and then deprived of mature T-cells) [85, 123]. Mice that lack both T-cell and humoral immunity were found to be remarkably resistant to infection with *Lm* at early time points, but were unable to control the infection in the long term. At the cellular level, neutrophils and macrophages are thought to be the principal mediators of the killing of *Lm*, and the depletion of granulocytes from mice markedly enhances their susceptibility to *Lm* infections [124]. Finally, *in-vivo* infections of *p47^{phox}* knock-out mice

emphasized the importance of the oxidative burst and the consequent production of ROS in the pathogen clearance: $p47^{phox}$ is one of the sub-units composing the NADPH-oxidase complex, therefore the generation of ROS in response to infections is impaired in these knock-out animals, and the susceptibility to *Lm* challenge is increased [125]. In the present study, it was chosen to investigate the role of MnSOD during *Listeria monocytogenes* infections because of its differences to the two previously described infection models. *Streptococcus pyogenes* and *Staphylococcus aureus* are preferential extracellular pathogens. The characteristic life cycle of *Listeria monocytogenes* inside macrophages and the importance of ROS production in bacterial challenge make it an interesting target for our investigation about MnSOD as a host resistance genetic factor during infection.

1.9 Conditional mouse mutants

Conditional mutagenesis offers the possibility to knock out genes of specific cell types or at a specific point in time. Thus, the effects of gene loss in a cell type or at a certain developmental stage of the organism can be examined. This kind of mutagenesis employs the Cre-loxP system and the Flp-FRT system.

Cre recombinase (causes recombination of the bacteriophage P1 genome) was originally isolated from the bacteriophage P1. Cre recombinase mediates the recombination between two loxP sequences [126]. The loxP sequence is an imperfect palindrome with two 13 bp inverted repeats, separated by an 8 bp non-palindromic sequence (spacer) [127]. The non-palindromic portion gives the loxP an orientation.

The recombinase-mediated deletion of the loxP-flanked region results in the loss of a plasmid containing the excised fragment and a single loxP site, while the other loxP is left on the genomic locus. This system has been proven to work very efficiently also in eukaryotic cells [128]. It can be used for deleting loxP-flanked genes or gene fragments[129] when the loxP

sequences are oriented in the same direction, or to induce the inversion of the floxed area, when the loxP orientation is opposite.

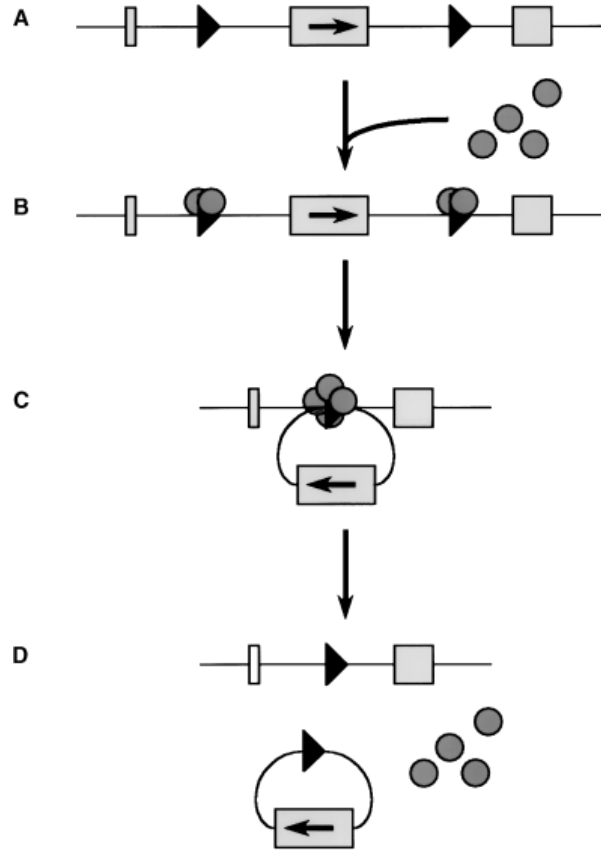


Figure 2: Cre/loxP system in gene targeting. Cre/loxP system requires the presence of two loxP sites flanking a DNA segment of interest and expression of Cre-recombinase. (A) The loxP sites are introduced *in vitro* into a DNA segment, which is then targeted to the endogenous locus by homologous recombination in ES cells. (B) Deletion of the floxed DNA element is achieved in cells expressing Cre-recombinase. Cre-recombinase assembles at the loxP sites as a dimer (B) and induces recombination between loxP sites as a tetramer (C). (D) Upon successful excision of the floxed DNA sequence, the genomic sequence is irreversibly modified. □ exons; ► loxP sites; ● cre-recombinase. Taken from [130].

Typical vectors for conditional mutagenesis contain (1) part of the target gene, with two homology regions upstream and downstream of the loxP sites, (2) two loxP sites placed in intronic regions, (3) the Neomycin gene as a selection marker which enables selection in bacteria and ES cells, and (4) a third loxP sequence or two FRT sequences that serve to delete the selection cassette after the identification of ES cells with homologous recombination. When using three loxP sequences, the selection marker is deleted by the Cre recombinase.

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This implies the disadvantage that not only the selection marker, but also the floxed gene section may be deleted. Identifying clones with a correct deletion of the selection cassette is considerably time-consuming in this case. With Flp-recombinase and its specific recognition sequences (Flp recognition targets), the two deletion events can be induced separately [131]. Flp-recombinase and the matching FRT recognition sequences were isolated from *Saccharomyces cerevisiae* [132, 133]. Similarly to loxP sequences, FRT sequences consist of two 13 bp inverted repeats, separated by an 8 bp spacer. Consequently, the FRT sequences are directional as well, with two possible orientations.

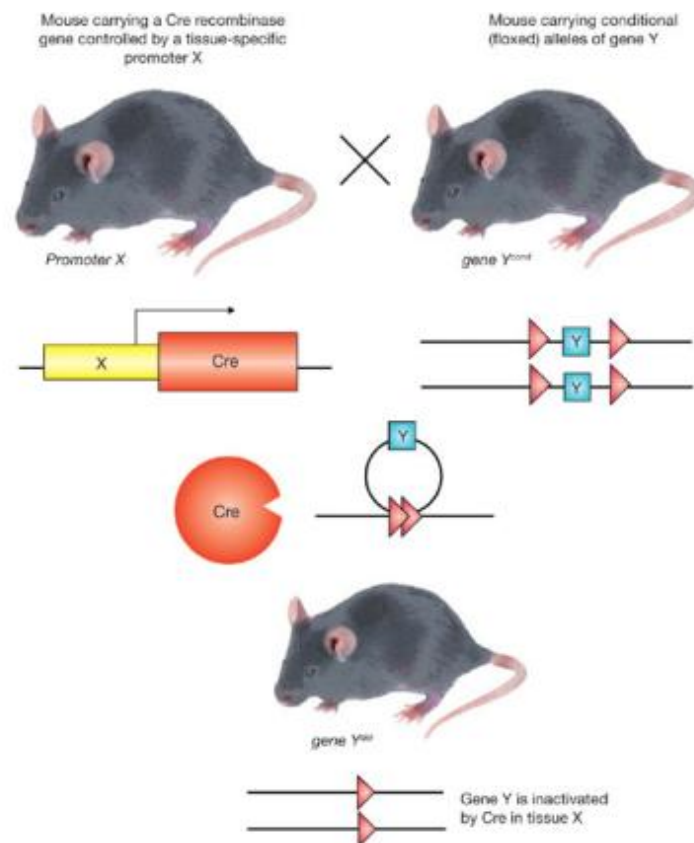


Figure 3: Conditional gene targeting. The gene cassette encoding Cre recombinase is usually engineered in a separate mouse strain. When Cre recombinase (red circle) is introduced, either as a transgene by crossing into a mouse line carrying the targeted gene locus or on a viral vector, the DNA between the loxP sites (red triangles) is removed, thereby inactivating the gene. Obtained from [134].

In the last decades, several transgenic mice containing the Cre recombinase gene downstream of cell-type specific or inducible promoters have been generated (the so-called Cre-lines). At present, the Cre-loxP system is mainly used for gene deletions, whereas the Flp-FRT system is used for deleting selection markers. Moreover, the deletion efficiency of the Flp-FRT system is lower than the Cre-loxP. Among the most important Cre lines used in immunological research, there are the CD19-Cre, CD4-Cre and LysM-Cre, in which the Cre recombinase is expressed downstream of promoters specific for B-cells, T-cells and Macrophages, respectively.

With respect to mouse lines in which the Cre-recombinase expression can be induced in a time-specific fashion, Mx-Cre line relies on the Mx promoter, which is functional only in presence of interferon- α : a single injection of interferon is sufficient to induce Cre and cause the deletion of the floxed fragment and subsequently to the inactivation of the gene. This thesis mainly employs LysM-Cre mice since macrophages and neutrophils, cells typically belonging to the innate immunity, play a major role in the analyzed infection models.

1.10 Generation of targeting vectors using the ET cloning technique

DNA modifications achieved through classical cloning techniques require restriction sites in a convenient position in order to do insertions and deletions and obtain a construct. The longer the construct, the more complicated the identification of unique restriction sites. This limits the possibilities for changing specific sections of a DNA.

ET cloning or recombineering is a relatively new technique that enables systematic mutation of mammalian DNA carried in *E. coli* [135]. This technique is based on the ability of the λ phage derived RecE and RecT [136-138] enzymes to promote homologous recombination of transformed DNA (i.e., PCR products carrying a mutated allele between the homology regions), with exogenous mammalian DNA carried extra-chromosomically in a vector or a

BAC (bacterial artificial chromosome). An equivalent system can be found in the α -phage with the Red α and Red β enzymes. This system can be used for *in vivo* mutagenesis of *E. coli* as well [139].

RecE and Red α are 5'-3' exonucleases [140], while RecT and Red β are proteins that bind single DNA strands. When both are present, they can mediate the overlapping of homologous DNA strands, transfer of single strands between different molecules, and strand inversion [136, 138]. The homologous recombination takes place more frequently when the DNA-binding protein is expressed in excess [141]. Figure 4 shows the underlying mechanism of the homologous recombination. The 5'-3' exonucleases RecE and Red α , respectively, recognize double-strand breaks, as can be found in linearized DNA. They then digest the DNA strands at the 5' ends. RecT and Red β can eventually bind to the resulting single strand protrusions, fostering the collocation of homologous strands, which leads to homologous recombination in the subsequent replication.

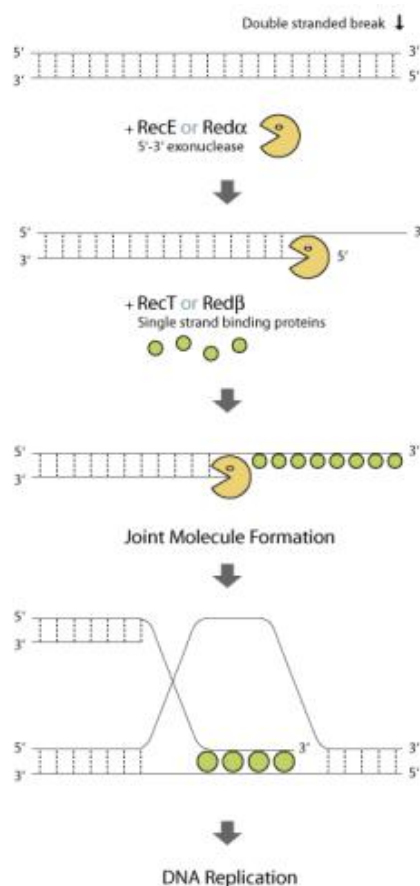


Figure 4: Red/ET Recombineering. The central step in Red/ET Recombineering is the crossover step between a targeting construct containing homology arms (hm) and the target which can be a gene locus on the *E. coli* chromosome or any other stretch of DNA in a BAC or plasmid vector. Obtained from www.genebridges.com.

The ability of the *in vivo* recombination can be transferred to various *E. coli* strains by means of a plasmid that codes for the RecE and RecT enzymes. Commercially available plasmids (i.e., Genebridges) contain information for the expression of the recombination enzymes, under the control of a promoter inducible with L-arabinose.

Due to the inducibility of the promoter, enzyme expression can be started at a designated time moment, thus avoiding unwanted recombination events. Moreover, plasmids have a temperature-sensitive replication starting point. This means that they will not be replicated at an incubation temperature of more than 30°C. At 37°C the plasmid will be progressively lost during cell division, avoiding any further unwanted recombination at the end of the recombination event. To enable the use of this method in all conventional *E. coli* strains, the

plasmids also contain the Red γ gene. Red γ encodes a λ -phage protein able to inactivate the *E. coli* RecBCD system [142, 143]. RecBCD is present in most of the *E. coli* strains and one of its purposes is to degrade linear DNA. Since ET cloning most often uses linear DNA in terms of PCR products, the activity of this enzyme complex has to be suppressed.

ET cloning can be used for various applications. In this thesis, ET cloning was used to generate a conditional knock-out transfection plasmid.

1.11 Research objectives

The present study is centered on conditional gene inactivation. Both the generation and the phenotypical analysis of conditional mouse mutants are described. The generation of conditional knock-out mice is an extremely time-consuming task, whose fulfillment can be negatively affected by several factors. Therefore, this thesis is divided into two parts:

PART I. By using the ET cloning technique, previously established by Dr. Anne Fleige in the Experimental Immunology group at the Helmholtz Centre for Infection Research (HZI), we planned a strategy to modify the endogenous locus of the *IL-12p40* murine gene contained in a BAC vector. This led to the creation of a targeting construct capable of recombining homologously within ES cells, which were subsequently injected into blastocysts in order to generate a conditional knock-out mouse line. In our knock-out strategy, we took into account the problems encountered in a previous PhD project concerning the creation of an *IL-12p40* conditional knock-out.

PART II. Macrophage and neutrophil specific MnSOD knock-out mice were obtained through the breeding of MnSOD^{fl/fl} to LysM-Cre mice. First, the specificity of the gene deletion was evaluated. In order to investigate a possible involvement of MnSOD in the

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protection of host organism components during bacterial challenge, in-vivo and in-vitro infections were performed. The three different bacteria used in the experiments were *Streptococcus pyogenes*, *Staphylococcus aureus* and *Listeria monocytogenes*. These were chosen in order to investigate various infection dynamics and kinetics.

2. Materials and Methods

2.1 Generation of the floxed IL-12p40 mouse

Mouse genomic DNA from a C57BL/6 bacterial artificial chromosome (BAC) clone containing the IL-12p40 gene in a pBACe3.6 vector (clone RP23-17E17 (RZPD) - www.ensembl.org -) was genetically modified using the ET cloning technique (Genebridges). First, a floxed Neo cassette carrying an additional EcoRI restriction site at the 3' end was inserted into intron 6 using the BAC modification kit (Genebridges) according to manufacturer's specifications.

Initially, a PCR was performed on the PGK-neo-loxP plasmid (Genebridges) with the primers 3'LOXs1 and 3'LOXas1 (for all primers, cf. Table 2). The deletion of the neo cassette was performed through transformation and induction of the PGK-Cre plasmid (Genebridges). Correct insertion was proved by PCR with the primers 3'LneoS1 (designed on the neo cassette) and 3'LneoAs1, resulting in a fragment of 1.1 Kbp only in the mutated sequence. The modified BAC was digested with BamHI. A 9 Kbp fragment containing the IL-12p40 gene was ligated to a pBluescript vector (pBIISK+, Promega) previously digested with BamHI. Correct ligation was screened by restriction analysis and sequencing. Plasmids were digested with EcoRI and BamHI, and the ones showing the expected bands were further processed. Subsequently, the second loxP site, followed by a FRTmut-flanked Neo cassette, was inserted into intron 3 using the BAC modification kit. Since the loxP was not present in the plasmid containing the cassette, it was inserted through an appropriate primer design. The two primers used to amplify the FRT-PGK-gb2-neo-FRT plasmid (Genebridges) were 5'LOXs1 and 5'LOXas1. Exploiting the presence of an EcoRI site in the FRTmut-flanked neo cassette, clones were screened for correct insertion by restriction analysis (BamHI and EcoRI

digestion). Sequencing of the mutated fragments was performed to further confirm successful and correct integration of the loxP sites and the additional Eco RI sites.

The correct functioning of the loxP and FRTmut sites was assessed by transformation of the targeting construct into Cre- and Flp-expressing *E. coli* strains (Genebridges, 234-FLP and 294-Cre, cat. # A101 and A111, respectively) and subsequent restriction analysis of the vector.

The construct was digested and linearized with SacII and transfected into five different ES cell lines. Genomic DNA from the G418-resistant clones was extracted and analyzed by Southern Blot or PCR to assess the correct homologous recombination of the 5' and the 3' loxP sites, respectively. In the first case, DNA was digested with EcoRI, blotted on a membrane and hybridized with the probe IL12_5': correct homologous recombination of the construct with the endogenous locus results in the detection of an 8.1 Kbp fragment, while the wt fragment's length is 7.7 Kbp. In order to detect the presence of the 3' loxP site at the correct site, and to avoid the amplification of fragments resulting from random recombination, the two primers for the PCR screening strategy, 3'LOXs1 and 3'WTas1, were designed on the loxP site and outside of the targeting construct homology region. The presence of a 1.5 Kbp fragment indicated successful integration.

ES cell clones carrying the mutated allele were then injected into blastocysts. Subsequently, chimeric offspring was tested for germ-line transmission.

2.2 Molecular biology techniques

2.2.1 Chemicals

Unless otherwise mentioned, all chemical products were purchased from Merck or Sigma-Aldrich Chemical Company.

2.2.2 Restriction Enzymes

All restriction analysis were performed with enzymes purchased from New England Biolabs together with their respective buffers, according to the manufacturer's specifications.

2.2.3 DNA isolation and gel purification

Plasmid DNA isolation was performed using Nucleo Spin or Nucleobond AX 500 kits (Macherey-Nagel). The purification of DNA from agarose gel or PCR products was performed using the QIAGEN gel-extraction kit.

2.2.4 Southern Blotting

Digested DNA samples were loaded on a 0.8% agarose gel and run by electrophoresis at 50V. in TAE buffer (see section 2.7.2) until a 1 cm separation between the 10 Kb and the 8 kb marker was obtained. Subsequently, the gel was incubated for 15 minutes in a 0.25N HCl solution, followed by 15 minutes in a 0.4N NaOH. The DNA was then transferred on a positively charged Hybond XL membrane (Amersham) via the alkaline capillary blot method.

2.2.5 Southern probe, radioactive labeling, and detection

The IL12_5' probe was generated by PCR and cloned into a TOPO4.0 vector (Invitrogen), and extracted by EcoRI digestion when needed. The probe consisted of a 750 bp DNA fragment obtained by PCR using the primers IL12_5's and IL12_5'as, and wt ES cell DNA as a template. The suitability of the probe was corroborated by the absence of repetitive elements, as confirmed by the analysis with RepeatMasker (A.F.A. Smit, R. Hubley & P. Green RepeatMasker at <http://repeatmasker.org>)

The efficiency of DNA cross-linking to the membrane was increased by one hour of incubation at 80°C following the blotting procedure. The membrane was equilibrated by

washing it in Church buffer at 60°C for one hour. Afterwards, it was prehybridized for at least two hours in QuickHyb (Stratagene) buffer at 65°C. Approximately 50 ng of the probe were labeled by PCR amplification with random primers and [$\alpha^{32}\text{P}$]-dCTP (Amersham Pharmacia Biotech) using the Ladderman Labeling Kit (Takara). After 45 minutes of incubation at 50°C, the PCR solution was purified on a centrifugation column (ProbeQuantTM G-50 Micro Columns; Amersham). 100 μl of fish sperm DNA were added. The probe was denatured at 95°C for five minutes and subsequently added to the prehybridization solution in which the membrane was incubated. The tube containing the membrane and the radioactive probe was incubated overnight in a rolling incubator at 65°C. On the following day, the membrane was washed twice for 20 minutes in Church buffer and then exposed overnight (O/N) to a Phosphorimager film. Radioactive emission of the film was acquired with the BASReader 3.14 software. The images were analyzed with AIDA 3.51 (both programs purchased at Raytest Isotopenmessgeraete GmbH).

2.2.6 Sequencing

DNA fragments were amplified and cloned into a TOPO4.0 vector prior to sequencing, which was performed at the HZI sequencing facilities using the primers M13 forward and reverse (Biospring).

2.2.7 Polymerase Chain Reaction (PCR)

ET cloning fragments, Southern probes, and mouse genotyping were performed by PCR using Taq-Gold polymerase and buffers from Roche. Volumes varied between 20, 25, and 50 μl . Melting temperatures depended on the sequence and the specificity of the primers. Elongation time was chosen based on the length of the expected product.

2.3 Cell culture

2.3.1 Murine embryonic stem (ES) cell lines

IDG32.F1 (C57BL/6 x SW129) and C57BL/6-derived Bruce4 ES cell lines were kindly provided by Dr. Ralf Kühn (GSF, Munich). The IDG32.F1 cell line was chosen because of its high rate of homologous recombination. The 129/Ola-derived ES cell strain IB10 was provided by the lab of Prof. Dr. Klaus Rajewsky, University of Cologne.

2.3.2 Murine embryonic feeder cells

These cells constituted the layer upon which ES cells were allowed to grow. These cells carry a G418 (neomycin) resistance gene and were obtained from IL-10 knock-out mice.

2.4 Media used in cell culture

2.4.1 Medium for murine ES cell culture

500 ml DMEM (glutamax, 4500 mg/l glucose, Na-pyruvate-free), 75 ml FCS (Gibco), 6 ml Na-pyruvate (Gibco), 6 ml penicillin/streptomycin (Gibco), 6 ml non-essential amino-acids (Gibco), 1.2 ml beta-mercaptoethanol (Gibco), and 1.2 ml Leukemia Inhibitory Factor (LIF; in-house production) were mixed under sterile conditions. When necessary, the medium was conditioned with 0.5 mg/ml G418 to enable screening of resistant clones.

2.4.2 Freezing medium

Prior to freezing, cells were re-suspended in a sterile filtered solution containing 50% FCS, 30% ES cell medium (w/o G418), and 20 % DMSO.

2.5 Cell culture techniques

2.5.1 Thawing of ES cells

Tubes of ES cells were quickly thawed in a 37°C incubator and then recollected into a Falcon tube, washed with pre-warmed medium, and centrifuged at 200g for five minutes. The pellet was then re-suspended in the appropriate volume of medium and seeded on EF covered culture dishes (Nunc).

2.5.2 Transfection of ES cells

ES cells were grown on a 10 cm dish at 37°C and 5% CO₂ until they reached approximately 70% confluency. ES cells were then rinsed once with PBS. 2 ml of 1x trypsin/EDTA were added to the dish, followed by five minutes of incubation at 37°C in order to detach them from the plate. The reaction was stopped by adding 8 ml of ES cell medium. The cells were harvested, centrifuged and re-suspended in 1 ml of ES cell medium. Viable cells were counted using a Neubauer-counting chamber and approx. 1×10^7 cells were transferred into an electroporation cuvette (Biorad) together with 10 µg of the linearized construct. Electroporation was set at 240 V and 475 mF using a Gene Pulser (Biorad). Afterwards, the cells were put at rest for 10 min. at room temperature (RT) and subsequently seeded onto feeder cells on 10 different Petri dishes. The medium was changed daily. After 48 to 72 hours (depending on the growth of the cells), the G418 selection was started at a concentration of 0.5 mg/ml. This took seven to ten days. After antibiotic selection, G418-resistant clones were aspirated and grown separately on a 96 well-plate (Nunc).

2.5.3 Isolation of ES cell clones

Clones integrating the neo cassette, in a random fashion or via homologous recombination, survived the G418 selection and grew as clones on the feeder cells layer covering the 10 cm

Petri dish. Plates were washed with PBS once and then re-filled with 10 ml of PBS. Individual ES cell clones were carefully removed in 10µl PBS using a 20µl Gilson pipette and then transferred to separate wells of a round bottom 96 well-plate containing 50µl 1X Trypsin/EDTA. After 20 minutes of incubation, the wells were filled with 100µl of G418 conditioned medium and subsequently split into three 96 well-plates. One of these plates was further expanded and split again in order to isolate a sufficient amount of DNA for PCR and Southern Blot analysis. The remaining two plates were stored at -80°C as master plates. The medium was changed daily. Gelatin-coated 96 well-plates were used for DNA isolation.

2.5.4 Freezing of ES cell clones

The two master plates were washed once with PBS and then incubated in 50µl 1X trypsin/EDTA per well for 5 min at 37°C. The reaction was stopped by adding 50µl ES medium. Afterwards, 100 µl freezing medium were added to each well. The plates were sealed with Parafilm and stored at -80°C to preserve them for further use.

2.5.5 Isolation of genomic DNA from ES cells

Cells were first lysed using proteinase K (Sigma) in lysis buffer at 54°C overnight. On the following day, the plates were cooled at room temperature (RT) for 30 min and 100µl of 100% ethanol were added in each well. The plates were incubated on a shaker for three hours at RT and then centrifuged at 3500g for 30 min at 4°C. The precipitated DNA was washed twice with 70% ethanol, dried at RT for 20 min, re-suspended in 30 µl TE buffer and finally incubated O/N at 54°C. A 1:10 dilution of each sample was used as a template for PCR screening. Digestion for Southern Blot analysis was achieved by adding 5µl 10X enzyme buffer, 0.5µl RNase A (10 mg/ml; MBI Fermentas) and 50 U restriction enzyme, followed by O/N incubation at 37°C.

2.6 Bacterial strains

2.6.1 *E. coli* strains

DH5 α chemocompetent bacteria were purchased from Invitrogen (cat. #: 12297016)

2.6.2 Cre-expressing bacteria and flp-expressing bacteria

E. coli 294-Cre and *E. coli* 294-Flp REF were purchased from Genebridges.

2.6.3 *Streptococcus pyogenes*

The group A streptococcus (GAS) KTL-3 strain was used in this study [98]. The stock cultures were stored in glycerole at -70°C and were subsequently cultured at 37°C in THY medium (see section 2.7.6). Bacteria were collected in mid-log growth phase, washed twice with sterile PBS, and diluted to the required inoculum. The number of viable bacteria was determined by counting colony forming units (CFUs) after diluting and plating on blood agar plates (Gibco) containing 5% sheep blood (Invitrogen, Karlsruhe).

2.6.4 *Staphylococcus aureus*

The SH1000 strain of *S. aureus* was used in this study [144]. Bacteria were grown to the stationary phase (15 h) at 37 °C with shaking (125 rpm) in brain heart infusion (BHI)-medium, collected by centrifugation for ten minutes at 3000 rpm, washed twice with sterile PBS, and adjusted to a concentration of 5×10^8 CFU/ml. Aliquots of the *S. aureus*-suspension were frozen at -80°C for further use. The bacterial suspensions were diluted with PBS to the required concentration. The number of viable bacteria (CFU) was determined after serial diluting and plating on blood agar plates.

2.6.5 *Listeria monocytogenes*

L. monocytogenes EGD (serotype 1/2a) stock cultures were maintained at -70°C and cultured overnight on a BHI-agar plate at 37°C. A single colony was inoculated in 5 ml of BHI broth and incubated overnight at 37°C. On the next day, the liquid culture was diluted 1:10 in BHI and cultured until mid-log phase. Bacteria were washed with PBS. Viable bacteria were counted using a Thoma chamber, and counting was confirmed by plating serial dilutions.

2.7 Media and buffers

2.7.1 LB Medium

LB medium was used for culturing *E. Coli*, and LB-agar to pour agar plates. 10 g bacto-tryptone, 5 g bacto-yeast extracts and 5 g NaCl were added to 950 ml of distilled water. The medium for LB-agar plates contained 15 g agar. The medium was conditioned with antibiotics at varying concentrations depending on whether the resistance was part of a high copy plasmid or a BAC: ampicillin at a final concentration of 100 mg/ml for high copy plasmid, and at 50 mg/ml for BACs; kanamycin at 50 mg/ml for high copy plasmids, and at 15 mg/ml for BACs; tetracyclin at 3 mg/ml for high copy plasmids; streptomycin at 50 mg/ml; and chloramphenicol at 15 mg/ml for BACs.

2.7.2 TAE (50x) for gel electrophoresis

242 g Tris, 57 ml 100% acetic acid and 100 ml 0.5 M EDTA with 1 l H₂O.

2.7.3 TE buffer

10 mM Tris/HCl (pH 7.4) and 1 mM EDTA (pH 8.0).

2.7.4 Church buffer

40 ml 1M NaHPO₄ (pH 6.8) and 50 ml 20% SDS were mixed with H₂O to give a 1:1 solution.

2.7.5 Tail buffer

200 mM NaCl, 100 mM Tris/HCl (pH 8.5), 5 mM EDTA (pH 8.0) and 0.2% SDS.

2.7.6 THY Medium

Todd-Hewitt broth was purchased at Oxoid (cat. #: CM0189) and supplemented with 1% yeast extract.

2.7.7 BHI medium

Brain Heart Infusion (BHI) medium was purchased from Difco (cat. #:237200).

2.8 Mouse strains

The mice were kept under specific pathogen-free conditions at the Helmholtz Centre for Infection Research (HZI) animal facility. All animal experiments were conducted in accordance with institutional guidelines. All mice were bred and maintained at the HZI animal facility.

B6;129P2-*Lzm-s2^{tm1(cre)Cgn}* (*LysMCre*) mice were on a pure B6 genetic background [145]. SOD2^{flox} mice were kindly provided by Prof. Dr. Scharffetter-Kochanek (University of Ulm, Germany) [146]. These mice were generated by the injection of 129/Ola ES cells into C57BL/6 (B6) blastocysts. Subsequently, mutant mice were backcrossed four times to B6 and further used for breeding and experiments. Due to the low number of backcrossings, these mice cannot be considered on a pure genetic background.

Breeding pairs were set up with $MnSOD^{fl/fl}$ X $MnSOD^{fl/fl}LysMCre$ mice. Therefore the generated offspring was in any case homozygous for the $MnSOD$ floxed allele. However, only 50% of the offspring carried the $LysMCre$ recombinase gene. Animals carrying the recombinase and consequently the cell-type specific mutation, are referred to as conditional knock outs or $LysMCre+$ mice. Mice without the $LysMCre$ recombinase are referred to as $LysMCre-$, and were used as wild type control.

2.9 Isolation of genomic DNA from mouse tails

The tip of a mouse tail was digested at 54°C overnight in 720µl tail-lysis buffer and 30µl proteinase K (10mg/ml). Cell debris and fur were separated by centrifugation at 13,000g for 10min. The supernatant was transferred into a new reaction tube containing 600µl isopropanol. To precipitate the genomic DNA, tubes were agitated and centrifugation was performed for five minutes at 13,000xg. The harvested DNA was rinsed with 70% ethanol, dried at RT for about 10 min and finally dissolved TE-buffer.

2.10 Mouse genotyping

Mouse genotyping was achieved by PCR. The floxed allele was detected using the primer pair $SOD2lox_s$ and $SOD2lox_as$ (cf. Table 2), while Cre was detected with the primer pair $cretot-1$ and $cretot2$ (cf. Table 2).

2.11 Cell sorting

2.11.1 Isolation of cells from the spleen

Mice were euthanized with CO₂. The spleen was excised and homogenized using a cell strainer with pores of 100µm diameter (BD Falcon, cat. #: 352360). Cells were re-suspended

in 5 ml of PBS/BSA (0.2%) and stored on ice. After centrifugation at 250g, the cell pellet was incubated for 10 min at room temperature with 3 ml of lysing buffer (BD Pharm Lyse™, BD Biosciences Pharmingen) for erythrolysis. The suspension was filled up to 50 ml with PBS/BSA, centrifuged and the pellet re-suspended in 5 ml PBS/BSA. To determine the cell number, a dilution of 1/50 was counted in a Neubauer counting chamber.

2.11.2 Antibodies staining for FACS analysis

FACS (Fluorescence Activated Cell Sorting) was used to sort cells into different cell types using fluorescent labeled antibodies against surface molecules. Cells were sorted into CD19+ B-cells, CD4+ T-cells, and F4/80+ macrophages via specific antibodies (cf. Table 1). An amount of 10×10^6 cells was used for the staining. Propidium iodide (2mg/ml in PBS/0.1%BSA) was added to each sample prior to sorting to discriminate between dead and living cells. Cell sorting was conducted with the MoFlo cell sorter (Cytomation). The purity of the sorted cell populations ranged between 90% and 99.9%. Sorting was performed both in *LysMCre-* and in *LysMCre+* strains. Each group contained four animals. All of the mice were at least 15 weeks old.

Table 1: Specification of antibodies

Antigen	Label	Specificity	Concentration	Cat-No	Supplier
F4/80	FITC	anti-mouse	0.1 mg/ml	MCA497PE	Serotec
CD4	PE	anti-mouse	0.2 mg/ml	553730	Becton Dickinson
CD19	APC	anti-mouse	0.2 mg/ml	550992	BD Biosciences Pharmingen

2.12 Bone marrow-derived macrophages and validation of the knock-out model

Three *MnSOD^{fl/fl}LysMCre+* and three *MnSOD^{fl/fl}LysMCre-* mice were sacrificed. Femurs were removed. Bone marrow was flushed out using 5 ml of cell culture medium (DMEM + 10% FCS + 100 µg/ml Penicillin/Streptomycin). Bone marrow was filtered through a cell strainer and washed twice. The cell suspension was then plated in cell culture medium containing 50 ng/ml of M-CSF in order to induce the differentiation of stem cells and the adhesion of macrophages to the plate. Cells were plated O/N at 37°C and 5% CO₂. After one day, the medium was changed, and M-CSF was added once again at the same concentration. The old supernatant was plated on a new Petri dish. At day four, medium was replaced with fresh one on both plates. On the seventh day after bone marrow extraction, differentiated macrophages were removed from the plate by incubation with 10 ml of 5 mM EDTA. DNA extraction was achieved through lysis buffer and proteinase K O/N incubation, followed by isopropanol precipitation (see section 2.9). Subsequently, genomic DNA was digested with BamHI for Southern Blot analysis according to a previously described protocol [146]. The Southern probe was kindly provided by Nicolai Treiber (University of Ulm, Germany). The specificity and efficiency of the deletion in macrophages collected from the *MnSOD^{fl/fl}LysMCre+* mice versus *MnSOD^{fl/fl}Cre-* mice was assessed by means of Southern Blot.

2.13 In-vivo infections

2.13.1 *Streptococcus pyogenes* (GAS KTL-3) infections

Mice aged 6-9 weeks (16 *LysMCre+* and 15 *LysMCre-*) were anesthetized via inhalation with Isofurane (Isoba, Essex Tierarznei) and infected subcutaneously with 5 X 10⁷ CFU of *S. pyogenes* KTL-3 in 100 µl of PBS at the right front flank in three independent experiments. Survival was checked twice a day. At day two, 10 µl of blood were sampled from the tail

vein, and serial dilutions were plated on blood agar plates. Colony-forming units were counted after 24 hours of incubation at 37°C.

2.13.2 *Staphylococcus Aureus* infections

Mice aged 8-12 weeks were inoculated with 4×10^7 CFU of *S. aureus* in 0.2 ml of PBS via a lateral tail vein. To determine the bacterial burden, infected mice were sacrificed by CO₂ asphyxiation at day one post-infection. The amount of bacteria was determined in the blood, liver, and kidney by preparing organ homogenates in 5 ml PBS and plating 10-fold serial dilutions on blood agar plates (kidney and liver), or by plating serial dilutions of blood collected directly from the heart. Bacteria colonies were counted after incubation for 24 hours at 37°C. Survival rates were checked once a day.

2.13.3 *In-vivo* infection with *Listeria monocytogenes*

Mice aged 10 to 14 weeks were injected via a lateral tail vein with two different amounts of bacteria, a sub-lethal dose (1×10^4 CFU) and the LD₅₀ (10^5 CFU) respectively. In the experiment with a sub-lethal dose the body-weight was monitored daily. The LD₅₀ injection was used to assess the survival rate, bacterial burden in liver and spleen (at day three post-infection) and for histological analysis.

2.14 Histology

Two days post-infection, mice were sacrificed by CO₂ asphyxiation. The peritoneal cavity was opened and the entire liver and spleen removed and fixed with 4% formalin for 48 hours. Tissues were processed and paraffin embedded using standard histological techniques. Sections were stained with H&E and were blindly scored for percentage of damaged tissue and neutrophil infiltration.

2.15 Cytokine assays

Two days after intravenous *Listeria monocytogenes* (*Lm*) infection, mice were sacrificed by asphyxiation. Blood was collected directly from the heart. The samples were left for two hours at RT and then centrifuged 10 min. at 2500 rpm. The supernatant was collected and centrifuged again in order to purify sera. Serum samples were analyzed on a Luminex 100 reader (Qiagen) using a mouse cytokine twenty-plex kit (Biosource), according to manufacturer's specifications.

2.16 Isolation and culturing of thioglycollate elicited peritoneal macrophages

Mice were injected 1 ml of thioglycollate intraperitoneally in order to attract monocytes in the peritoneal cavity and activate macrophages [147]. Five days after the injection, mice were sacrificed by cervical dislocation and the skin was removed from the abdomen. Peritoneal exudates cells (PECs) were collected by injecting 10 ml of ice-cold PBS/FCS (1%) into the peritoneal cavity and then recollecting into a Falcon tube. After a washing step in PBS/FCS, the pellet was re-suspended in 5 ml of ACK buffer and incubated for five minutes at RT, in order to allow erythrocyte-lysis. PECs were counted and aliquots of 3×10^5 cells were plated on 24 well-plates (Nunc) and incubated O/N in cell culture medium. To increase the activation state of macrophages and to enable them to challenge *Lm* infection, the medium contained 10U/ml of Interferon- γ (IFN- γ).

2.17 In-vitro infection with *Listeria monocytogenes*

Thioglycollate-elicited macrophages were used for in-vitro infection experiments to evaluate differences in the ability to challenge *Listeria monocytogenes* between MnSOD knock-out (*LysMCre+*) and wild-type macrophages (*LysMCre-*). Three different multiplicities of infection (MOIs) of *Lm* were used: 0.1, 1, and 5. After 16 hours of IFN γ stimulation, cells

were washed twice with PBS, and three different dilutions of a *Lm* liquid culture in the mid-log growth-phase were put on top of the adherent cells. Plates were centrifuged 5 min. at 150g, and then incubated 30 min. at 37°C, to allow bacterial uptake inside the phagocytes. Medium containing left-over bacteria was removed, the plate washed and gentamycin-enriched medium was added to ensure the survival of *Lm* only inside phagocytes. Macrophages were incubated for 30 min, subsequently conditioned medium was removed and gentamycin-free medium added. Immediately after incubation in gentamycin conditioned medium, and after one and three hours, the concentration of *Lm* in the wells was determined as follows. Wells were washed once with PBS and 500 µl of lysis solution (0.5% Saponin in PBS) were added and incubated for 3 min. Aliquots of the lysis solution were serially diluted and plated on BHI-agar plates. Colonies were counted after 24 hours of incubation of the BHI-agar plates at 37°C, 5% CO₂. Collected data were analyzed as CFU/well and were subsequently normalized to the average CFU at time 0 to evaluate the clearance ability regardless of the different uptake capacity.

In order to determine the cytotoxic effect of the pathogen challenge on macrophages, LDH release was measured using the Cytotox96 kit (Promega, Germany, cat. #: G1780), according to manufacturer's instructions. Briefly, 50 µl of the supernatant were collected at 0, 1,4, and 24 hours after infection. Uninfected cells for each time point were used to measure lactate dehydrogenase (LDH) spontaneous release.

2.18 Intraperitoneal *Listeria monocytogenes* infection and FACS

Mice were injected 5 X 10⁶ CFUs of *Lm* intraperitoneally (i.p.). One hour after infection, PECs were collected as described in section 2.17. Cells were incubated for five minutes in erythrolysis buffer and then re-suspended in 3 ml of PBS/FCS (1%). 200 µl aliquots were transferred to a V-bottom 96 well-plate for further staining. First, FcγR was blocked by 5'

incubation with CD16/32 antibodies (1:50 dilution). Cells were washed twice in Annexin-V Binding Buffer (BD bioscience). F4/80-FITC, CD11b-APC, and Annexin V-PE, were purchased at BD bioscience and titrated for optimal staining conditions, corresponding to 1:200, 1:400, and 1:100 dilutions, respectively. Surface markers were stained by 30 min incubation at RT in Annexin-V buffer. After staining, cells were washed twice and re-suspended in 300 µl of Annexin-V binding buffer. Propidium iodide (1:10.000) was added to the samples immediately before loading them into a FACS-CANTO (BD-Bioscience) apparatus.

2.19 Primers

All primers were purchased from Biospring (Frankfurt am Main, Germany) (cf. Table 2).

Table 2: PCR primers (All primers are represented in the 5' 3' orientation)

Amplification of the PGKNeoLoxP plasmid	
3'LOXs1	GGCGCAACGCAATTAATG ATGGCGCAACGCAATTAATG
3'LOXas1	AACTGATGGCGAGCTCAGACGAA <u>TTCA</u> ACTGATGGCGAGCTCAGAC C
First insertion checkup	
3'LneoS1	TTCATCGACTGTGGCCGGCT
3'LneoAs1	TTAGTAAACTCCAACAGAAA
Amplification of the FRTmut-flanked neomycin resistance gene (loxP site underlined)	
5'LOXs1	CTGTTTGCATCTTTTCTTGTTTCA <u>ATAACTTCGTATAGCATACATTA</u> <u>TACGAAGTTAT</u> CTGCGATCGCCTAGGGGTAACC
5'LOXas1	TGATTAGGAAGGGAAGACCTTGGAAAAGCTGGCGCGCCGTCGC
Amplification of the Southern Blot probe IL12_5'	
IL12_5's	AACTTGTCAAGGTCACATAAC
IL12_5'as	CATATAGTCAAGACAAGTACAAG
MnSOD mice genotyping	
SOD2lox_s	GCTGTCATTGCTTTTAACTGC
SOD2lox_as	TGCCAGATGTCACCTTAAAG
Cretot1	ACGACCAAGTGACAGCAATG
Cretot2	CTCGACCAGTTTAGTTACCC

PART I: Generation of the IL-12p40 conditional knock-out mouse

3. Results for PART I

3.1 Conditional targeting vector design

The design of a good targeting vector is a crucial and probably the most difficult task for a successful strategy. IL12-p40 consists of 8 exons, and the gene sequence was obtained in the Ensemble genome database and analyzed with Vector NTI (Invitrogen) to establish which region of the gene should have been excised for an efficient knock-out. The latter is usually achieved via a frameshift creating early stop codons (often leading to digestion of the immature RNA), or by removing essential functional domains of the expressed protein. It was established that a deletion of the region including exons from 3 to 6 would efficiently inactivate the gene for the following reasons: (1) exons from 3 to 6 make up $\frac{3}{4}$ of the whole cDNA, and (2) due to the induced frameshift, only the first 31 of the remaining 78 amino acids possibly translated, remain intact. The rest of the amino acids are different due to the induced frameshift. Therefore, the two loxP were placed in intron 2 and intron 6 (cf. Figure 5).

Transfection of ES cells with a targeting vector very often results in a random integration of the construct, whereas homologous recombination occurs less frequently.

The isolation of ES clones containing the construct was realized by adding a FRTmut-flanked neomycin cassette downstream of the loxP in intron 2, while discrimination between random versus homologous recombined clones was assessed by a Southern Blot and PCR strategy. Two EcoRI sites were inserted into the construct next to the loxP sites in order to create DNA fragments differing in length from the wild type, which were detectable with a labeled probe recognizing a region located outside of the targeting construct (cf. Figure 9).

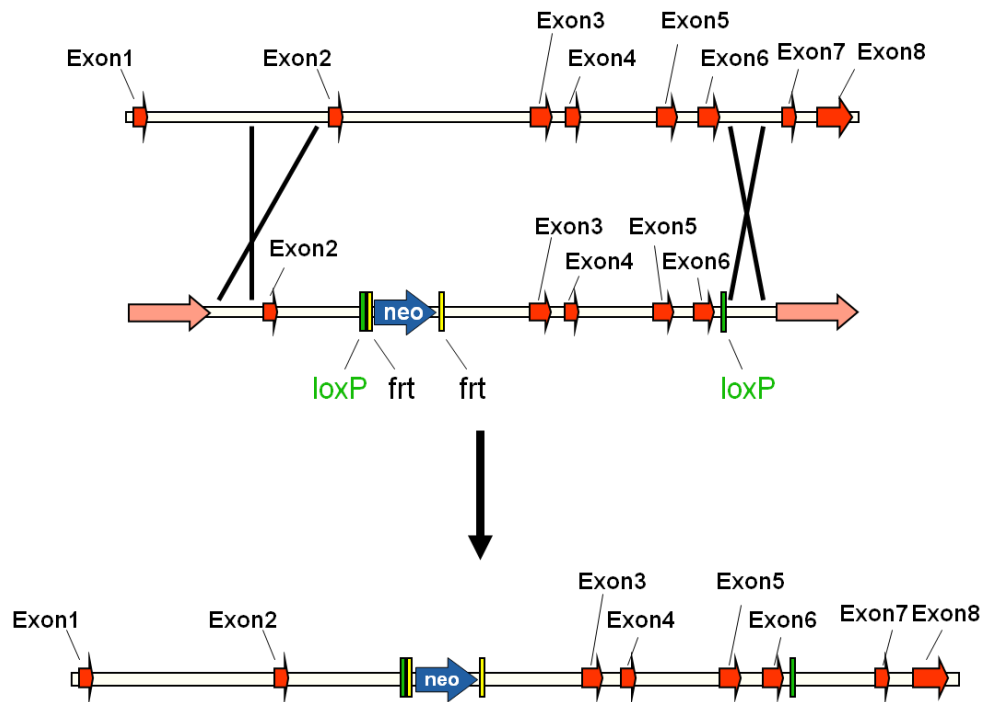


Figure 5: Targeting strategy. Homologous recombination between the endogenous locus (top) and the targeting construct is rendered possible by the homology regions upstream of the 5'loxP and downstream of the 3'loxP.

3.2 Generation of the targeting vector

Every ET cloning step was preceded by the transfection of the ET plasmid into *E. coli* cells carrying the BAC with the IL12-p40 gene. First, a Neo cassette flanked by two loxP sites and the additional EcoRI restriction site was inserted into intron 6. Correct insertion was proven by the growth of clones on Neomycin conditioned LB agar plates, followed by PCR analysis (cf. Figure 6).

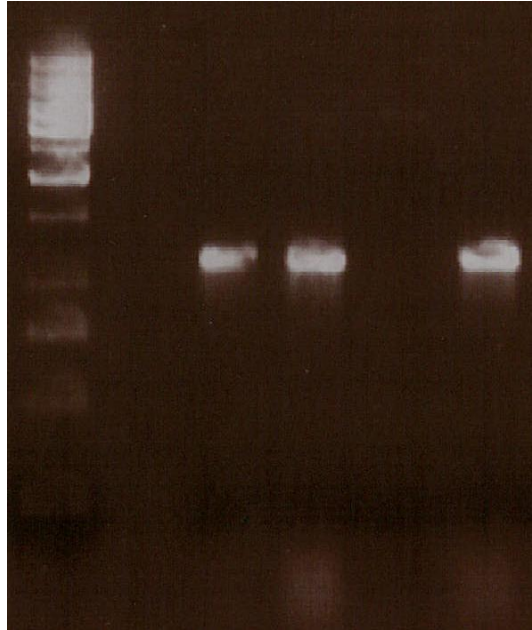


Figure 6: LoxP-flanked neo cassette insertion. After the first ET-cloning step, five different BACs were isolated. Only BACs containing the cassette display a 1.1 Kbp band (lanes 2,3 and 5).

Using primers within the Neo cassette and downstream of it, PCR amplification of the wt BAC sequence did not reveal any fragment, while the mutated locus showed a fragment of 1.1 Kbp.

A BAC portion of 9 Kbp containing the IL-12p40 gene was ligated to a pBSIISK⁺ vector, using the neomycin resistance for selection of transformed clones. Successful ligation was confirmed by sequencing.

In the second ET cloning reaction, another loxP preceded by an EcoRI site and followed by an FRT-flanked Neo cassette was inserted into intron 2. Restriction analysis was performed with EcoRI in order to prove a 1:1 ligation (Figure 7). Vector NTI software predictions (Invitrogen), indicated the presence of 4 fragments deriving from EcoRI digestion, whose lengths are approximately 5.3 Kbp, 3.5 Kbp, 2.6 Kbp, and 700 bp (cf. Figure 7).

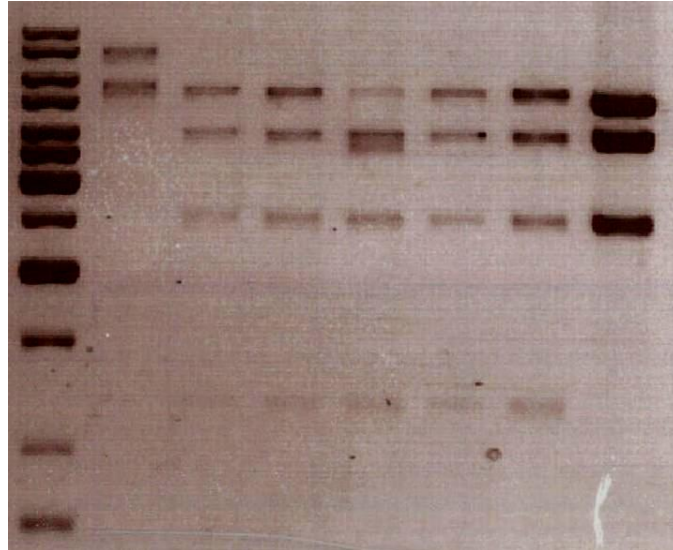


Figure 7: Restriction analysis of the targeting construct. Seven different plasmids were digested with EcoRI. Expected bands were observed in lanes 2, 3, 5, and 6. The seventh lane shows a control digestion of the targeting vector before the last insertion step was performed. Only plasmids shown in the fifth and in the sixth lanes were further processed.

3.3 Analysis of the targeting vector

Transfection plasmids showing the appropriate restriction patterns were further sequenced in HZI facilities. This confirmed the presence of the introduced elements. The plasmid was then transformed into *E. coli* strains expressing Cre and Flp recombinase to verify the correct function of the loxP and FRT sequences. After *in vitro* Cre-mediated deletion, the plasmid was digested with BamHI, leading to the expected products at 4.3 Kbp and 4 Kbp. (cf. Figure 8). Similarly, after Flp-deletion the plasmids were digested with BamHI, leading to fragments of 8.1 Kbp and 4 Kbp (Figure 8). In both cases the length of the fragment was consistent with what expected by software simulation.

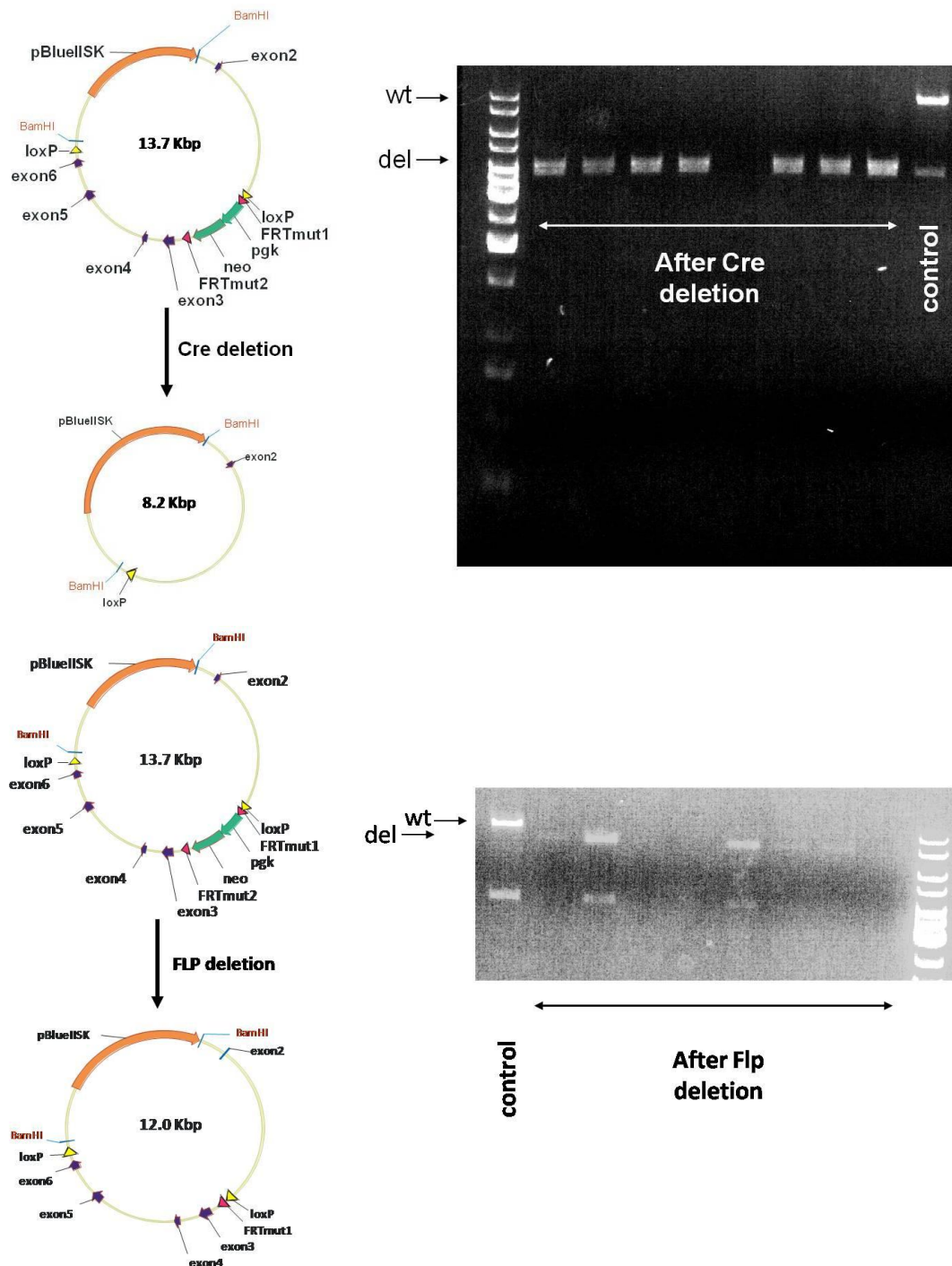


Figure 8: Restriction analysis subsequent to Cre and FLP mediated excisions. results in the loss of a 5.5 Kbp fragment . Digestion of the intact vector displays two bands at 9.8 Kbp and 3.9 Kbp (control). The 3.9 Kbp fragment is not affected by the recombinase-mediated deletions. After Cre-mediated deletion of a 5.5 Kbp fragment, a new band of 4.3 Kbp is present (upper right gel picture). FLP-mediated recombination results in the loss of a 1.7 Kbp fragment, as shown by the 8.1 Kbp band.

3.4 ES cells transfection and screening for homologous recombination

10 µl of the targeting construct were linearized by SacII digestion and transfected into five different ES cell lines: IDG32.1 F1, IB101, BALB/c, Bruce4, and E14. A total of approximately 2500 clones were found to be G-418 resistant. These were then grown on 96 well-plates to allow for DNA preparation and homologous recombination screening. The latter consisted of two strategies based on Southern Blot and PCR for the 5' and the 3' loxP, respectively. Only 5 out of the 2500 isolated clones showed the bands indicating for the wild type and the mutated allele (Figure 9).

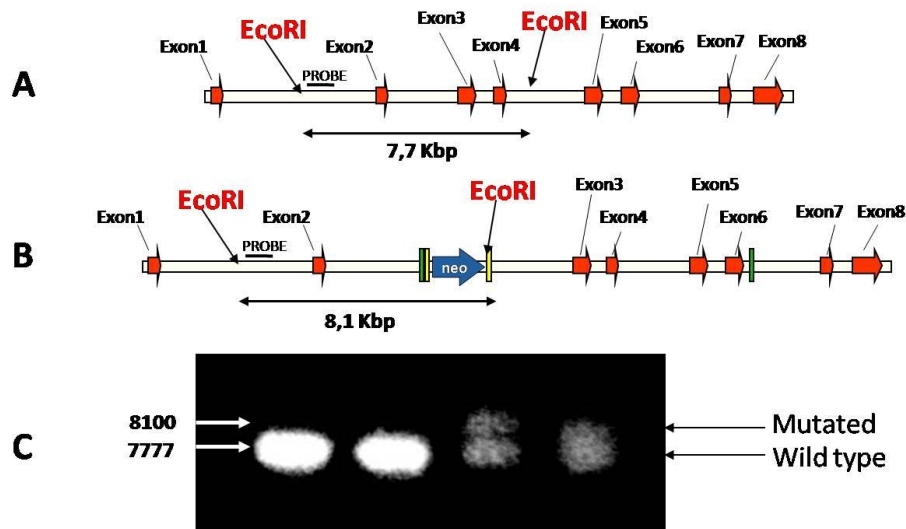


Figure 9: Southern Blot strategy. Hybridization of the probe with EcoRI-digested wt DNA (A) and mutated DNA (B), results in the detection of two fragments of different length (C).

PCR was used to detect homologous recombination of the loxP contained in intron 6. By means of one primer matching on the loxP site and the other primer outside of the targeting vector homology region, a single clone (IL12c117) carrying the correct mutation was identified among the five clones previously isolated.

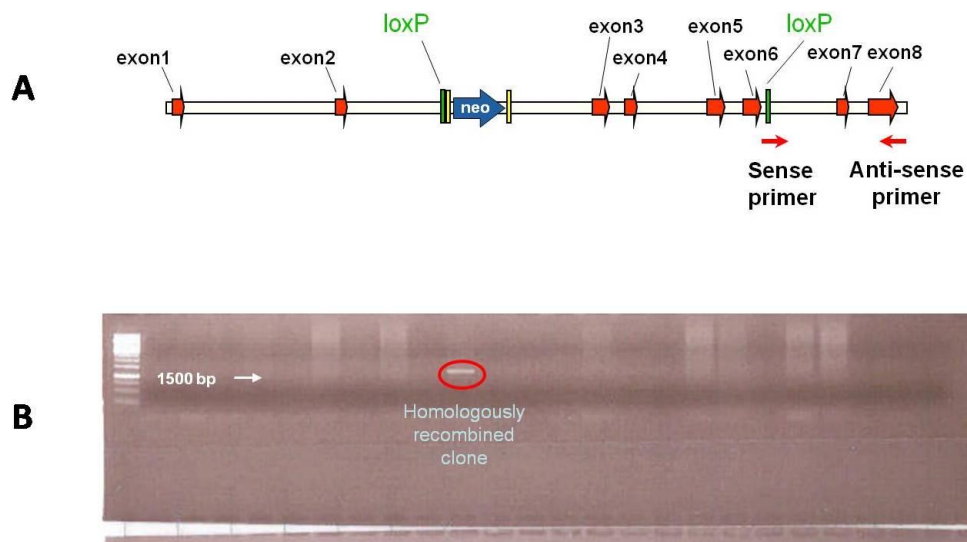


Figure 10: PCR strategy for the detection of homologously recombined clones. Sense primer of the PCR screening (3'loxP2) is specifically annealing to the loxP sequence (A), whereas the antisense primer is located outside of the targeting construct homology region. Therefore, the presence of a 1.5 Kbp PCR product proves the presence of a homologously recombined clone (B).

3.5 Injection of the positive clone into blastocysts

IL12cl17 was expanded and injected into blastocysts of BALB/c mice. The injection resulted in the birth of 3 chimeras, which were bred with C57BL6 mice to be tested for germ-line transmission. Two of the three chimeras died, while the third one remained sterile.

4. Discussion of PART I

4.1 DNA engineering using ET cloning

The generation of the IL-12p40 conditional knock-out consisted of three phases: generation of the targeting construct, ES-cell transfection, and blastocysts injection of homologously recombined clones. Although the generation of the construct and the identification of homologously recombined clones were performed in a reasonable amount of time, no transgenic mouse was generated. ET cloning has previously been used in our group for the generation of two conditional knock-out targeting vectors . This method was chosen because it enables the manipulation of large DNA fragments, such as the IL-12p40 targeting construct described in the above chapter. Given the long distance between the loxP sites (5.5 Kbp) and the length of the homology regions (4.1 Kbp at the 5' end, and 1.2 at the 3'), ET cloning allowed for the engineering of a large genomic region while avoiding the drawbacks of conventional cloning. Conventional cloning relies on restriction and ligation steps, and the ease of use depends on the frequency of restriction sites along the fragment of interest. Considering the length of the targeting vector (12 Kbp) and the lack of unique restriction sites, standard cloning techniques would have proven inconvenient. ET cloning, on the other hand, permits homologous recombination in *E. coli* between a PCR-amplified fragment containing a 20 bp homology arm on each side and a target sequence. This procedure offered the right flexibility in choosing the insertion regions and in determining the length of the targeting construct homology arms.

Although Genebridges states that a construct can be generated within three weeks, the generation of the construct took approximately six months. Compared to the time used previously by other members of our group to generate targeting vectors, there is a positive trend with respect to time-consumption. Nevertheless this time could be strongly reduced in several ways. First, all the PCR-amplified fragments used in the different recombination steps

could be generated in advance, thus saving time in the time intervals between the screenings and the transfections. Furthermore, optimization of the electroporation phase showed that the time to make the cells competent should be as quick as possible and the temperature kept low. Performing the washing steps in a cold room (4°C) and on ice improved the efficiency of the recombination. The use of a BAC (single copy vector) instead of a high copy plasmid also improves the time-consumption, since it avoids several purification and isolation steps. Moreover, integrations might be confirmed via appropriate PCR strategy instead of restriction analysis. Genebridges supplied our lab with a plasmid whose sequence differed from the version described in the manual. Thus, amplification of that particular plasmid was impaired. This turned out to be the main problem during the entire generation process.

4.2 Transfection and screening

Conditional gene targeting of the IL-12p40 locus had already been attempted in our group by Dr. A. Fleige [148]. The targeting vector she used had most of its features in common with the one described in the present thesis. The position of the loxP sites and the length of the homology arms was the same. The only substantial difference concerned the position of the FRT-flanked neo cassette, that in the first generated construct was placed downstream of the 3'loxP site, whereas in the described construct is placed in intron 2. The above mentioned research project was unsuccessful because of the failure in the ES cell transfection step. This suggested that the presence of a large insert such as the Neo cassette (1.7 Kbp) next to the short homology arm impairs homologous recombination. Moreover, since intron 2 is approximately four times larger than intron 6 (5.4 Kbp vs 1.4 Kbp), placing the Neo cassette in intron 2 is less likely to have an effect on IL-12p40 expression in the absence of Cre-recombinase. Homologous recombination in ES cells is a rare event and has been shown to be influenced by several parameters, such as the length of the homology regions, and the origin

of the ES cell line used for the transfection [23, 24, 149]. Only five clones out of 2500 G-418 resistant ones had undergone homologous recombination at the 5' end. These five clones all belonged to the Bruce4 ES cell line, a C57BL/6 derived cell line. The results of our targeting confirm previous observations of the BL/6 IL12p40 locus being the best for mutation purposes. This might be due to epigenetic factors such as chromatin rearrangement, which possibly allow access to the gene only in BL/6 derived ES cell lines. Taking into account only the Bruce4 transfection for the above mentioned reasons, the rate of correct homologous recombination is still very low (1 positive clone out of 430 = 0.23%). It could be argued that this is due to the short length of the 3' arm of homology. Indeed, the latter allowed a high throughput transfection-screening strategy by PCR. Compared to Southern Blotting approaches, PCR results less expensive and time-consuming. However this is a minor gain and does not repay the loss in homologous recombination efficiency. In the future it will be important to take into account that only C57BL/6 derived ES cells allow homologous recombination in the IL-12p40 locus, and a construct with longer homology arms should be generated.

PART II: Phenotypical analysis of macrophage and/or neutrophil specific MnSOD mouse mutants

5 Results for Part II

MnSOD conditional mouse mutants for macrophages and/or neutrophils were generated in order to investigate MnSOD involvement in bacterial infection models. Two groups of mice, *MnSOD^{fl/fl}LysMCre+* (*LysMCre+*, conditional knock-out group) and *MnSOD^{fl/fl}* (*LysMCre-*, control group), were infected with *S. pyogenes*, *S. aureus*, and *L. monocytogenes* in order to investigate the role of MnSOD expression in macrophages and neutrophils in the context of *in-vivo* and *in-vitro* infections. Prior to infection, the efficiency and specificity of *LysMCre*-driven gene-deletion was evaluated by Southern Blot analysis.

5.1 LysMCre efficiently catalyzes the excision of the floxed fragment in macrophages of *LysMCre+* mice

To examine whether *LysMCre*-driven deletion is specific and efficient, genomic DNA of bone marrow-derived macrophages (BMDM) was isolated from *LysMCre+* and *LysMCre-* mice. Southern Blot analysis revealed good efficiency of the Cre-mediated deletions only in BMDM belonging to the *LysMCre+* group (Figure 11), thus confirming the reliability of the Cre-loxP method for the inactivation of the MnSOD gene. The length of the “floxed” is approximately 2.6 Kbp. When *LysMCre* deletion takes place, a BamHI restriction site is lost, the length of the recombined fragment increases up to 7.8 Kbp. The excision of the floxed fragment is not 100% efficient, as shown by the presence of a light flox band at 2.6 Kbp in the *LysMCre+* BMDM lane. This means that the excision of the floxed fragment catalyzed by *LysMCre* recombinase is efficient, and the *MnSOD^{fl/fl}* x *LysMCre* model is suitable for studying MnSOD deficiency in macrophages and/or neutrophils.

It has already been demonstrated that the excision of the third exon results in the inactivation of the gene product [150, 151]. Hence, the deletion showed in the picture implies the inactivation of the MnSOD gene.

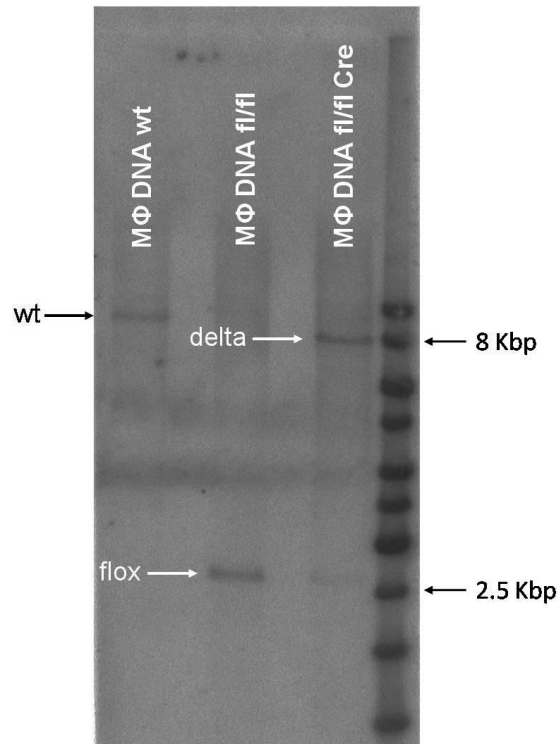


Figure 11: LysMCre-driven excision in *LysMCre+* BMDM. DNA was extracted from macrophages isolated in wt, *MnSOD^{fl/fl}*, and *MnSOD^{fl/fl}LysMCre+* mice. In the wt lane an 8.5 Kbp band is visible. The MnSOD fl/fl lane contains a single 2.6 Kbp band. In the Cre+ lane, the delta band (7.8 Kbp) is present, together with a flox band of very low intensity.

T- and B-cells DNA from *LysMCre+* and *LysMCre-* mice was isolated after cell-sorting. DNA digestion, electrophoresis, blotting, and hybridization with the radioactive probe were performed at the same conditions described for the DNA isolated from macrophages. However, no band was detectable in these samples.

5.2 *Streptococcus pyogenes* infections

5.2.1 MnSOD deficiency in macrophages and neutrophils does not influence susceptibility to GAS KTL-3 infection

21 *LysMCre*⁺ and 20 *LysMCre*⁻ mice were infected subcutaneously with 1 to 3 X 10⁷ CFU of GAS KTL-3 in four independent experiments. Mice health status was checked daily and survival curves were derived. Bacteremia was measured two days post-infection in blood samples collected from the tail.

In the first experiment, *LysMCre*⁻ mice were found to be more susceptible to the infection (Figure 12-A). Consistently with this finding, bacteremia in the *LysMCre*⁻ group was higher than in the *LysMCre*⁺ group (Figure 13-A). Due to the low number of animals in the experimental groups, the difference was not statistically significant. In the second experiment, *LysMCre*⁺ mice were found to be more susceptible than the *LysMCre*⁻ mice (Figure 12-B), and also bacterial levels in the blood were higher in the *LysMCre*⁺ group (Figure 13-B). In the third experiment, susceptibility to the infection was similar in the two experimental groups (cf. Figures 12-C and 13-C).

In the fourth and last experiment, *LysMCre*⁻ mice appeared once again more susceptible to the infection than *LysMCre*⁺ mice, although bacteremia levels measured in the two groups were substantially identical (cf. Figure 12-D and 13-D). Taken together, these results show no difference between *LysMCre*⁺ and *LysMCre*⁻ mice in response to subcutaneous GAS KTL-3 infections (cf. Figure 14).

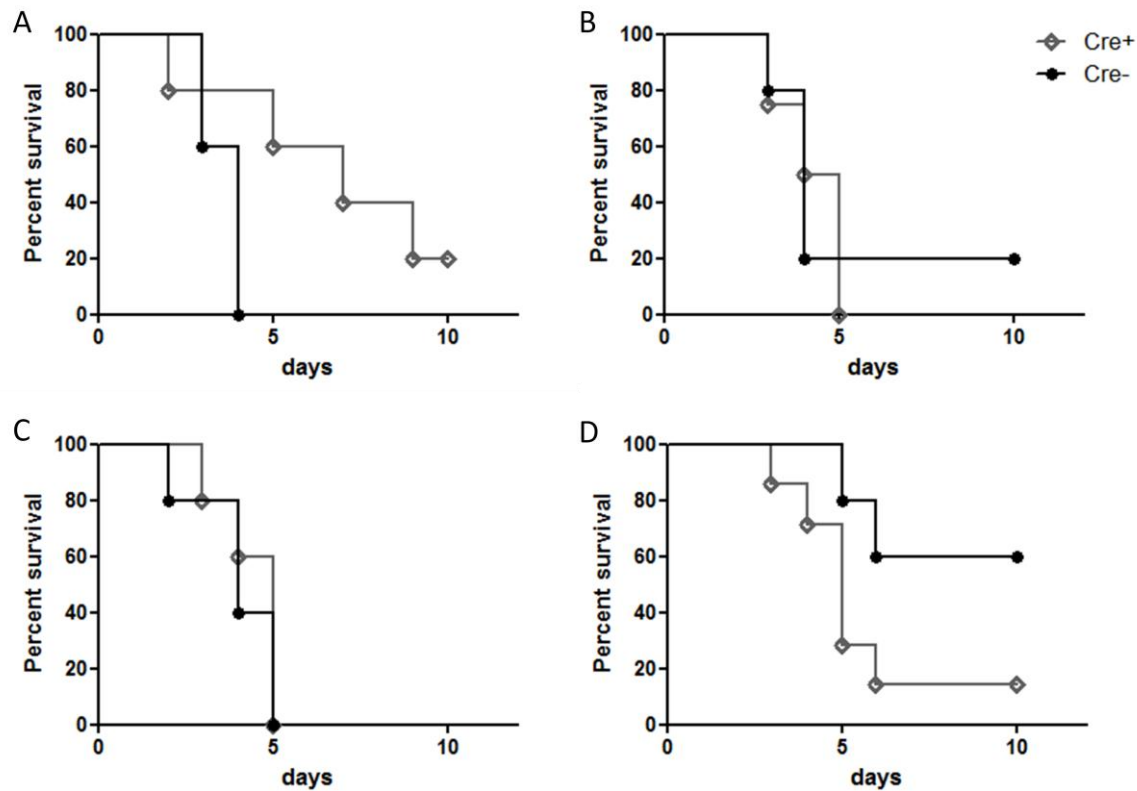


Figure 12: Susceptibility to GAS-KTL-3 infections. Difference in susceptibility to GAS KTL-3 infections between *LysMCre+* and *LysMCre-* mice was highly variable in four independent experiments. (A) 100% of *LysMCre-* mice died within four days after the infection, while 20% of *LysMCre+* mice was able to challenge the infection and survive. (B) Contrary to the first experiments, GAS KTL-3 infection was lethal for all the *LysMCre+* mice, whereas 20% of the *LysMCre-* survived the infection. (C) Similar survival trends between the two groups were found in the third experiment. (D) 60% of the *LysMCre-* group and 10% of the *LysMCre+* group were able to survive the infection. None of the above mentioned differences was statistically significant.

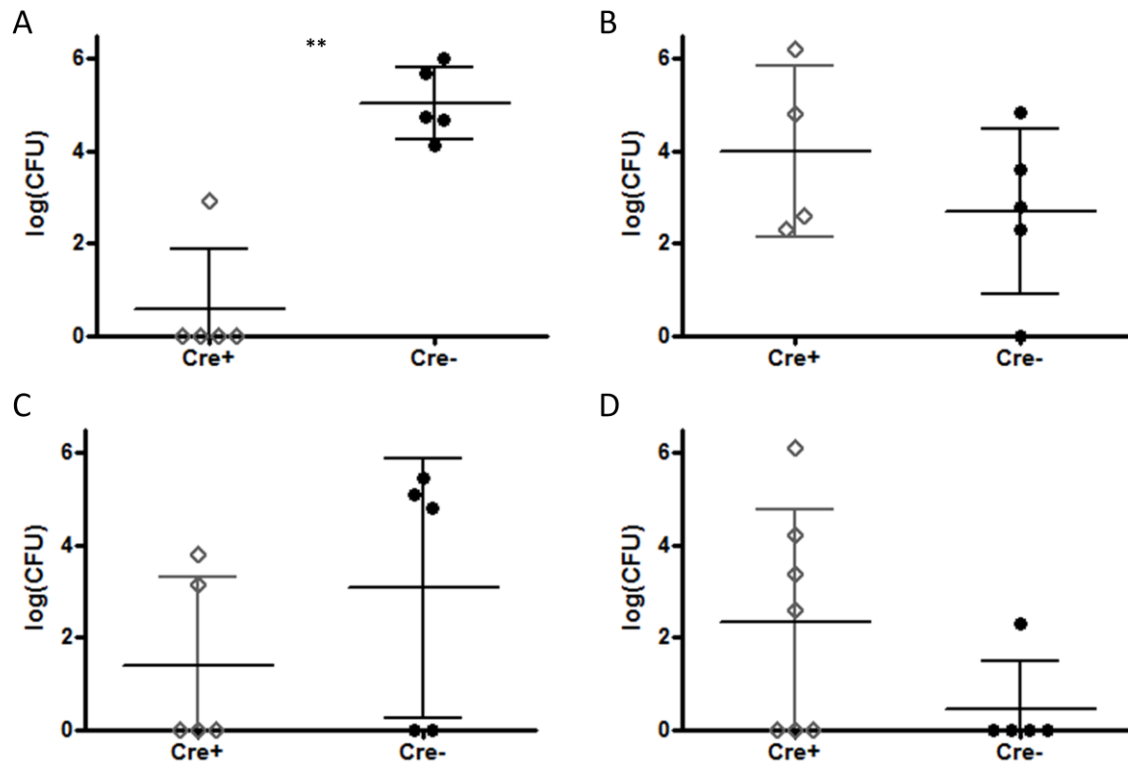


Figure 13: Bacteremia. GAS KTL-3 CFU in blood samples collected from *LysMCre+* and *LysMCre-* mice were similar in three out of four experiments (B-C-D). (A) bacteremia in *LysMCre-* mice was 10.000 times higher than *LysMCre+* mice (** p<0.01)

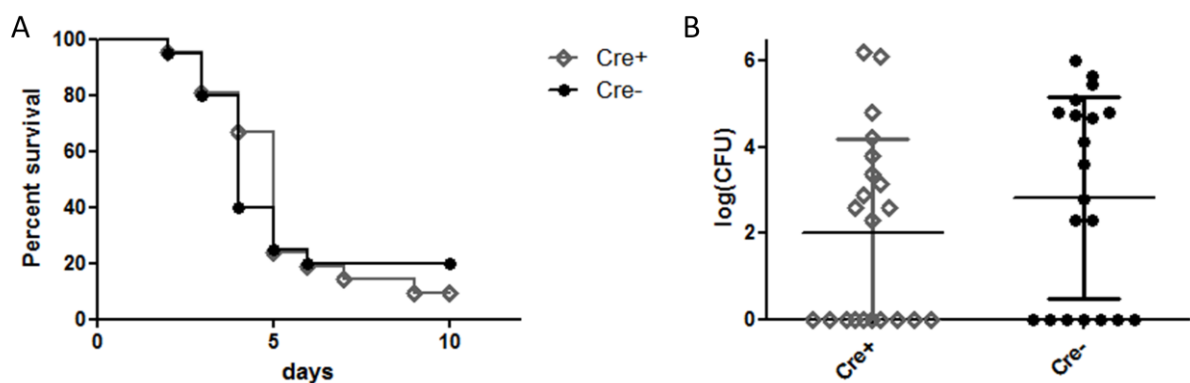


Figure 14: Cumulative results of GAS KTL-3 subcutaneous infection. (A) *LysMCre+* and *LysMCre-* mice display no difference in susceptibility to GAS KTL-3 subcutaneous infection as evinced from the similar survival rate curves. (B) In both groups bacteremia ranges from 0 to 10^6 CFU/ml of blood. No statistically significant difference is present between *LysMCre+* and control mice.

5.3 *Staphylococcus aureus* infections

Infections of several in-bred mouse strains with *S. aureus* showed that C57BL/6 mice are very efficient in controlling bacterial growth and clear the infection [144], leading to high survival rates. SW129 mice exhibit an intermediate phenotype, as they are not completely resistant to such infections ([144]). Hence, the mixed background of *MnSOD^{fl/fl}LysMCre* mice was not expected to play a major role in this infection model, as in the case of *S. pyogenes*.

5.3.1 Susceptibility to *Staphylococcus aureus* infection is not influenced by MnSOD expression in macrophages and neutrophils

12 *LysMCre*⁺ and 10 *LysMCre*⁻ mice were inoculated intravenously with 4×10^7 CFU of *S. aureus* in 0.2 ml of PBS via a lateral tail vein. In both groups, mice started to die three days after infection, thus displaying an intermediate phenotype between C57BL/6 and SW129 parental strains (Figure 11).

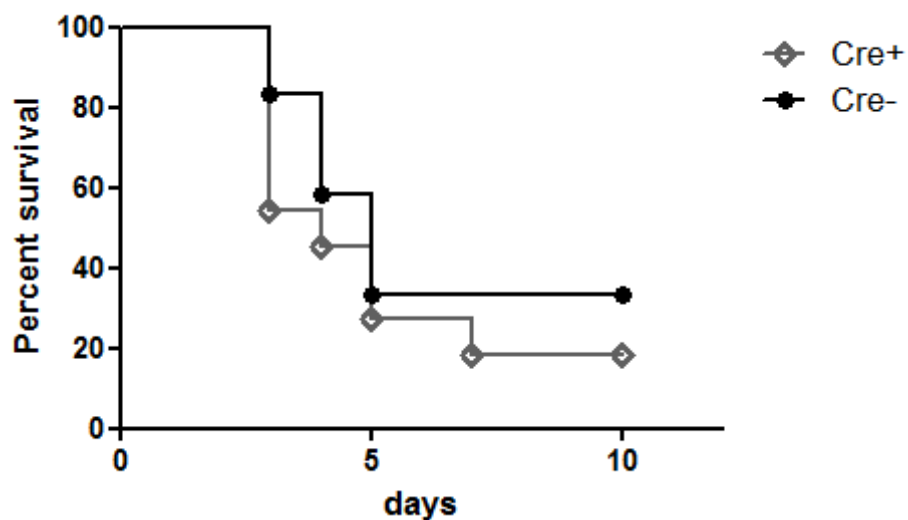


Figure 15: Survival rate. *S. aureus* infection of *LysMCre*⁺ and *LysMCre*⁻ mice results in similar survival rates.

LysMCre⁺ mice were slightly more susceptible to bacterial challenge, however this difference was not statistically significant.

Bacterial levels in organs and blood were more clustered than in the streptococcal infections, suggesting a lower influence of the genetic background on the outcome of the infection with *S. aureus*. Nevertheless, no difference between *LysMCre*⁺ and control mice could be found (Figure 12). Taken together with survival rates, these results prove no major role for MnSOD over-expression as a protection factor in this infection model.

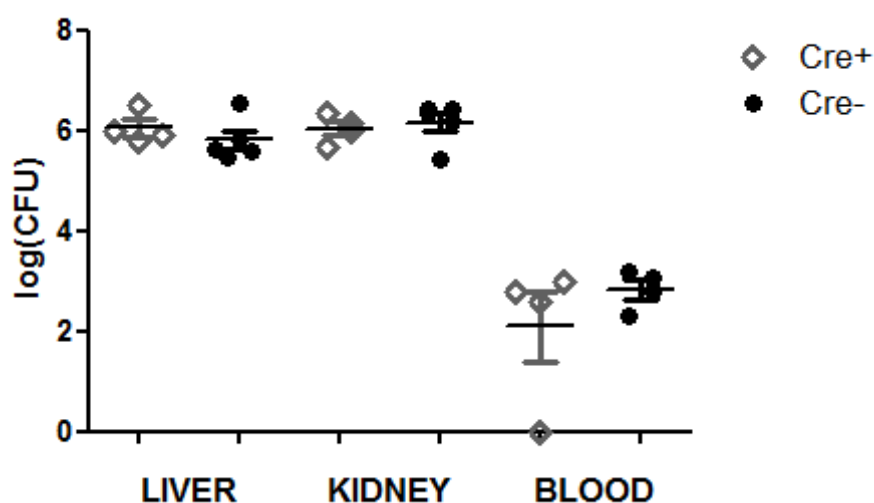


Figure 16: Bacterial burden in blood, liver, and kidney homogenates. One day after *S. aureus* intravenous infection, there is no significant difference between MnSOD conditional knock-out mice and control mice.

5.4 *Listeria monocytogenes* infections

5.4.1 Sub-lethal infection

A sub-lethal dose of *L. monocytogenes* was injected intravenously in order to assess the course of the infection with respect to the genetic background of the animals. The weight of the mice was measured before the infection (w_0), and every day the percentage was calculated by dividing the registered weight by w_0 . Both groups displayed weight loss starting from day

two post-infection. Between day three and four, mice of the control group began to heal, while the *LysMCre*⁺ group mice displayed a drop in their health status at day four. At this time point, two mice from the *LysMCre*⁺ group died, and the recorded values decreased to 10% of the original weight. The observed difference in weight loss between the two groups was statistically significant (Figure 13).

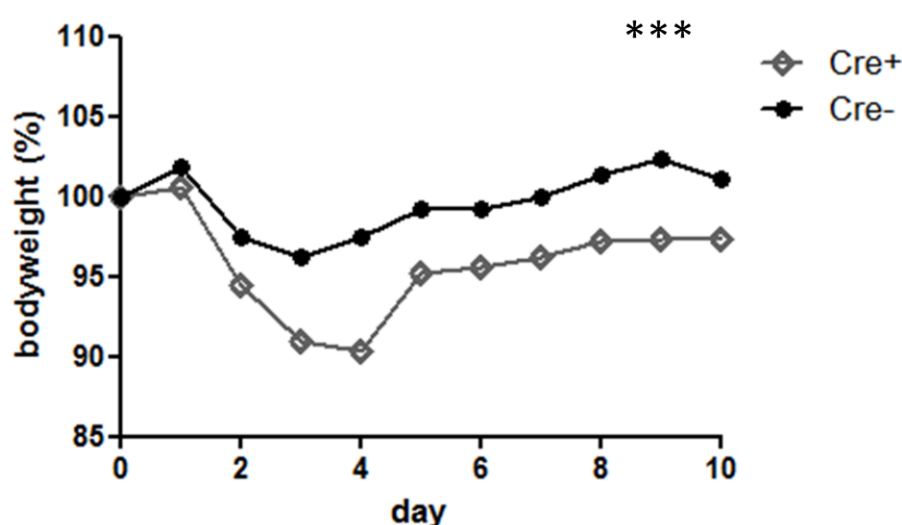


Figure 17: Bodyweight analysis. After intravenous infection with *Lm*, *LysMCre*⁺ mice display a higher bodyweight loss along a time period of ten days. (***) $p < 0.001$

5.4.2 Survival experiments

It has been shown that female mice are more susceptible to *Listeria* infections [99]. For this reason, male mice and female mice were infected in two independent experiments with 10^5 CFU of *L. monocytogenes* (male mice) and 4×10^4 CFU (female mice). The amount of bacteria used for the intravenous infections corresponds approximately to the LD₅₀ for the C57BL/6 strain. In both experiments, MnSOD conditional knock-out mice were significantly more susceptible to the infection than controls (Figure 14). In the experiment involving male animals and a higher bacterial inoculum, 50% of the control individuals died by day eight

post-infection and the remaining half recovered and survived until day 15, while all mice of the *LysMCre+* group died by day seven.

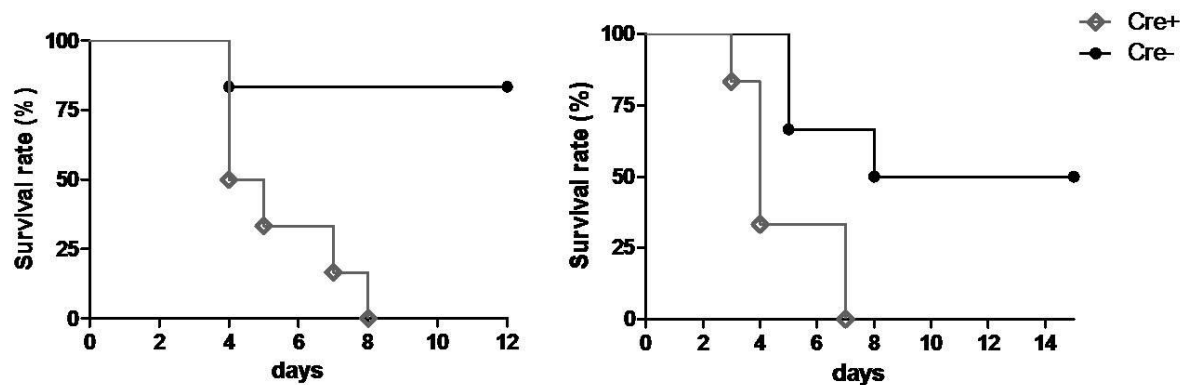


Figure 18: *Listeria monocytogenes* intravenous infections in female mice (left) and male mice (right). Both female and male *LysMCre+* mice were significantly more susceptible than the sex-matched controls ($p=0.0055$ and $p<0.05$, respectively).

Combining the results obtained in sex-matched experiments, *MnSOD^{fl/fl}LysMCre+* mice were found to be significantly more susceptible to intravenous infection with *L. monocytogenes* than *LysMCre-* control mice (Figure 19).

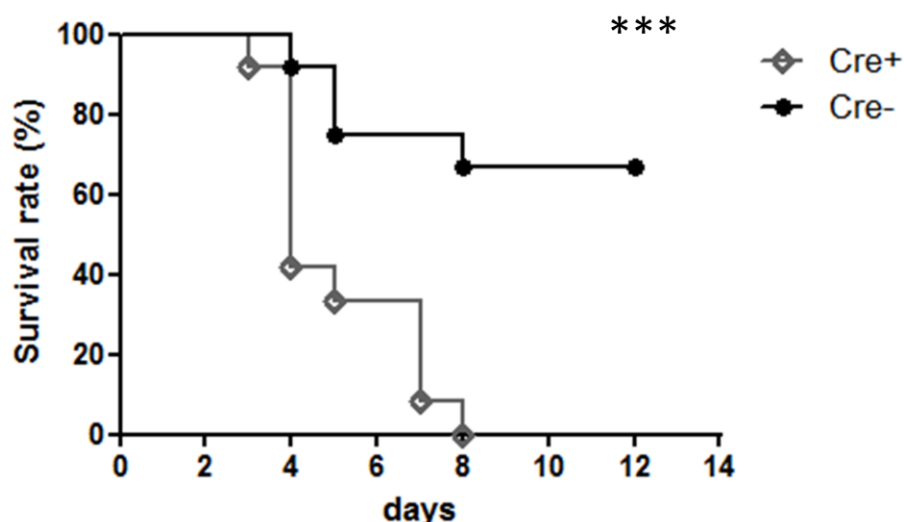


Figure 19 Cumulative survival rates results. Infection with a LD₅₀ inoculum of *Listeria monocytogenes* was lethal for 100% of the *LysMCre+* mice, whereas 65% of the control animals were able to challenge infection. This difference was statistically significant (***, $p<0.001$).

5.4.3 A higher *Listeria monocytogenes* burden is found in liver and spleen of conditional MnSOD knock-out compared to wild-type mice

Liver and spleen are the main accumulation organs after systemic *Listeria monocytogenes* infection [119-121]. In the intravenous infection model, bacteria are directly released in the bloodstream, and two days after inoculation it is possible to evaluate the amount of bacteria infecting the organs. Bacterial burden in liver and spleen homogenates, as well as in blood samples, was measured in two independent experiments (cf. Figure 20). Five-fold higher levels of *Lm* were found in livers of *LysMCre*⁺ mice compared to the controls. Similarly, bacterial burden in spleens of MnSOD conditional knock-out mice was two times higher than in the controls. Both differences were statistically significant. No difference was found between blood samples.

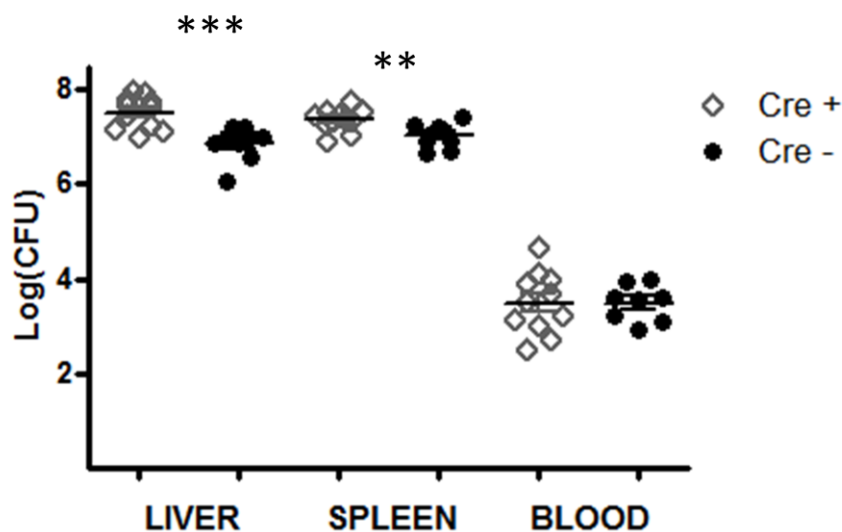


Figure 20: Organ burden two days after *Lm* infection. Higher amounts of bacteria were found in liver and spleen samples of *LysMCre*⁺ mice than in the controls. (**p<0.01; ***p<0.001)

5.4.4 Conditional MnSOD deficiency influences *Listeria monocytogenes* induced damage in liver and spleen

Liver and spleen are the main targets of *Listeria monocytogenes* after bacteria reach a systemic distribution. In the intravenous infection model, access to these organs takes place immediately after injection. Livers and spleens of mice infected with 2×10^5 CFU were removed for histological analysis 48 hours after infection. H&E staining allows the detection of damaged tissue and mononuclear infiltration *foci*. Each slide was scored for percentage of damaged tissue. Livers of *LysMCre*⁺ mice presented severe multifocal mononuclear infiltrate, consisting of macrophages and few neutrophils (Figure 21-A). The number and dimensions of these inflammation *foci* was reduced in livers deriving from *LysMCre*⁻ mice (Figure 21-B). Moreover, only in infiltrates of *LysMCre*⁺ mice livers it was possible to distinguish a central area of liquefying necrosis, revealing a higher damage and tissue disruption.

Spleen sections from both groups displayed multifocal liquefying necrosis, recognizable by the disruption of the typical splenic tissue organization (Figure 21-C D). In most cases, such formations were wider in spleens extracted from *LysMCre*⁺ mice. Spleen slides were scored on the percentage of necrotic tissue over the total area of the slide (Figure 22). Statistical analysis evidenced a significantly higher damage in liver slides of *LysMCre*⁺ mice. Difference in spleen scores was not statistically significant.

The number and dimensions of these inflammation *foci* was reduced in livers deriving from control mice. Moreover, only in infiltrates of *LysMCre*⁺ mice livers it was possible to distinguish a central area of liquefying necrosis, revealing a higher damage and tissue disruption (Figure 21).

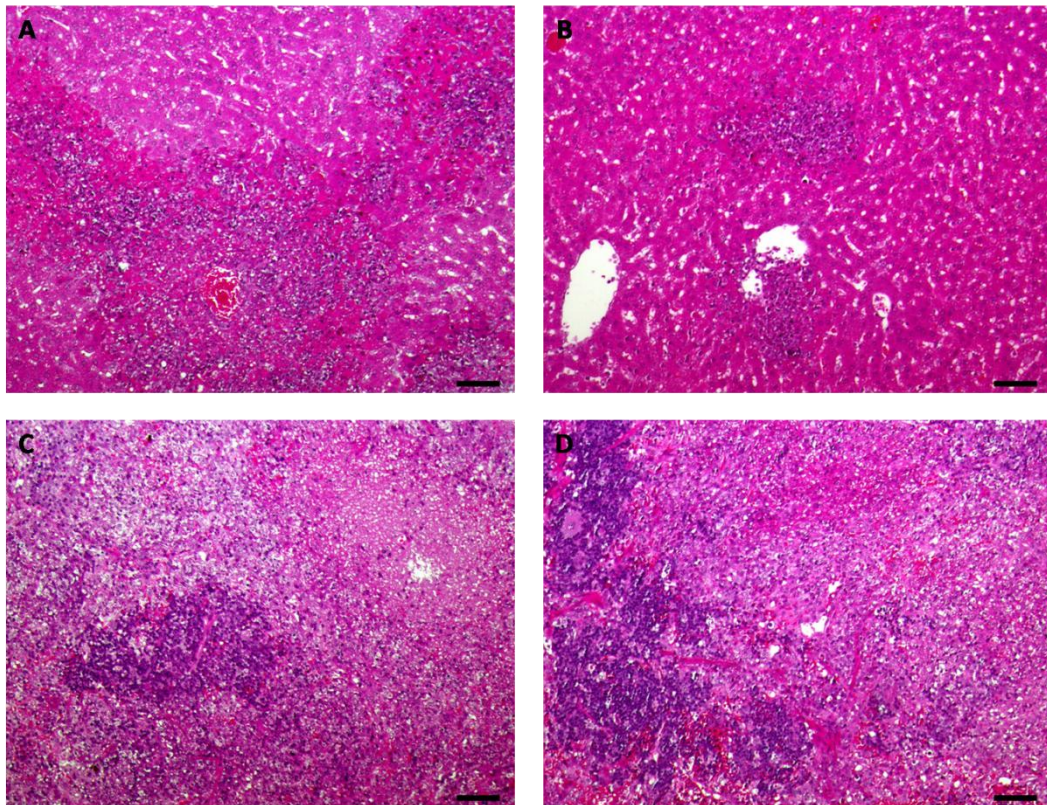


Figure 21: H&E staining of liver (A-B) and spleen (C-D) sections from *Listeria monocytogenes* infected mice at day two. Mononuclear cells infiltrates are larger in liver slides of *LysMCre*⁺ mice (A) than *LysMCre*⁻ (B). A central area of liquefying necrosis is present in A, next to the blood vessel. C-D: typical organization of the splenic parenchyma is visible in the lower-left corner (dark purple). In the upper-right portion of the *LysMCre*⁺ spleen slide (C), a wide area of liquefying necrosis surrounded by macrophages is present. In the control slide (D), a smaller area is visible in the same portion of the slide. = 200 μ m.

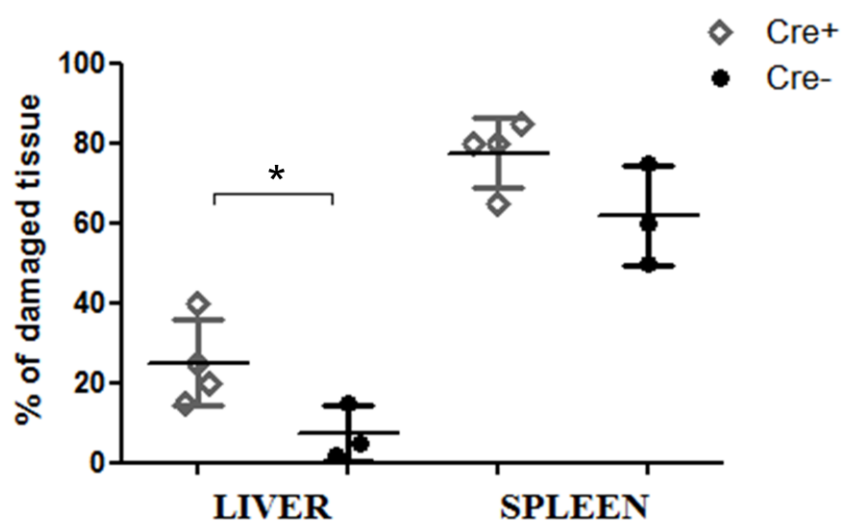


Figure 22: Spleen and liver histological score of *LysMCre*⁺ and *LysMCre*⁻ mice. Both in the liver and the spleen slides, the percentage of damaged tissue is higher in *LysMCre*⁺ organs than in controls. This difference is statistically significant only in the liver slides. (*p<0.05)

5.4.5 MnSOD knock-out macrophages uptake higher amounts of bacteria and are impaired in challenging *Listeria monocytogenes* infections

The ability of macrophages isolated from *LysMCre*⁺ and *LysMCre*⁻ mice in challenging *Lm* infections was tested in two experiments with different multiplicity of infection (MOI).

In the first experiment, 10^6 cells were infected with approximately 10^5 CFU of *Lm* (MOI=0.1). Infected cells were lysed after incubation with the pathogen (time 0), and after one and five hours. Bacterial uptake in MnSOD knock-out macrophages was ten times higher than in the wild-type ($p<0.01$, cf. Figure 23). One hour after infection, *LysMCre*⁻ macrophages were able to clear the pathogen. On the contrary, MnSOD knock-out macrophages were able to reduce the bacterial burden after one hour but were unable to control the infection, as shown by the increase of CFU measured in the cells after five hours. Data normalized to the initial uptake confirmed that *wild-type* macrophages are more efficient in clearing the infection after one and five hours (cf. Figure 24).

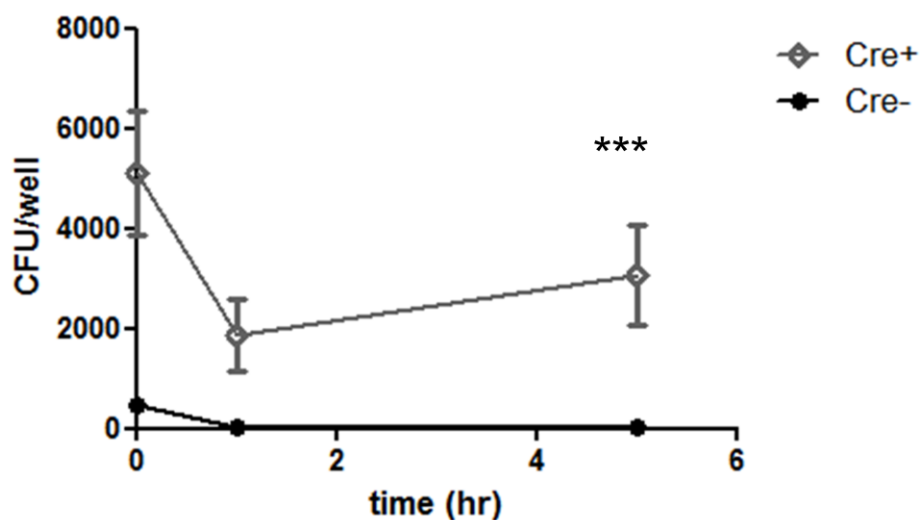


Figure 23: In-vitro macrophage infection with *Listeria monocytogenes* (MOI=0.1). Ten-fold higher uptake of *Lm* in *LysMCre*⁺ versus *LysMCre*⁻ macrophages is shown at time 0. A higher amount of *Lm* CFU is found inside *LysMCre*⁺ cells compared to the control. (***) $p<0.0005$)

In the second experiment 3×10^5 macrophages were infected with 3×10^4 CFU of *Lm* (MOI=1). The amount of bacteria surviving inside the cells was measured at zero, one, and three hours

post-infection. Bacterial uptake was three times higher in MnSOD knock out macrophages in comparison to wt macrophages (cf. Figure 25).

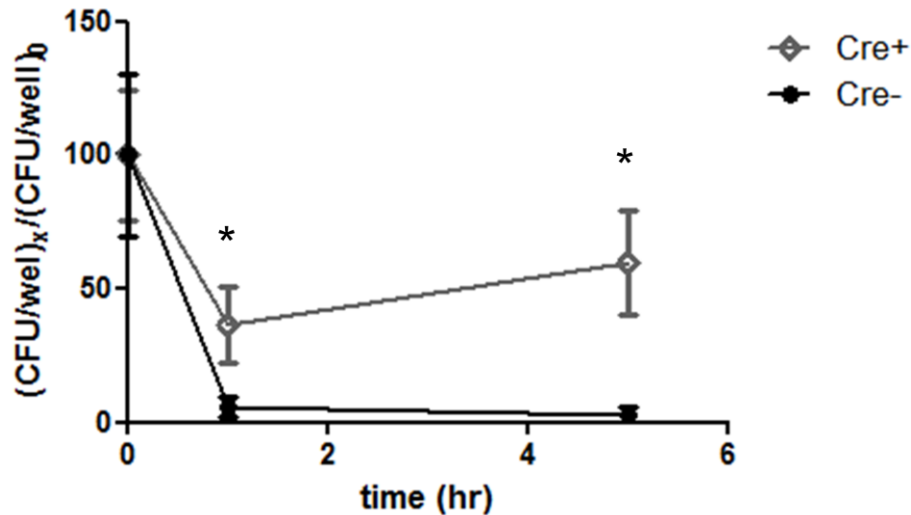


Figure 24: Normalized CFU data (MOI=0.1). One and five hours post-infection, the percentage of uptaken bacteria still surviving inside phagocytes is significantly higher in *LysMCre+* macrophages than in the control. (* $p < 0.05$)

Moreover, this experiment confirmed what was found in the first experiment, since the amount of bacteria inside *LysMCre+* cells was higher than in *LysMCre-* macrophages at all time points. Nevertheless, *wild type* macrophages were not able to clear the infection as seen in the previous experiment with a lower MOI. Normalized data showed no significant difference at any time-point (cf. Figure 26). Both in *LysMCre+* and in *LysMCre-* macrophages, there was a reduction of the bacterial load after one hour. In any case, after three hours the amount of CFU raised up to approximately 100% of the value measured immediately after the infection. Therefore, no significant difference was observed between the two groups in challenging the infection.

Cytotoxicity caused by the pathogen was assessed in a third in-vitro infection experiment (MOI=5). Cell supernatants were analyzed for the presence of LDH (fold increase over uninfected) at zero, one, four, and 24 hours post-infection. Four hours after infection, *wild*

type macrophages were releasing significantly higher levels of LDH than the *LysMCre+* (cf. Figure 27). After 24 hours, the cytotoxic effect measured in *LysMCre+* cells was significantly higher than in the control.

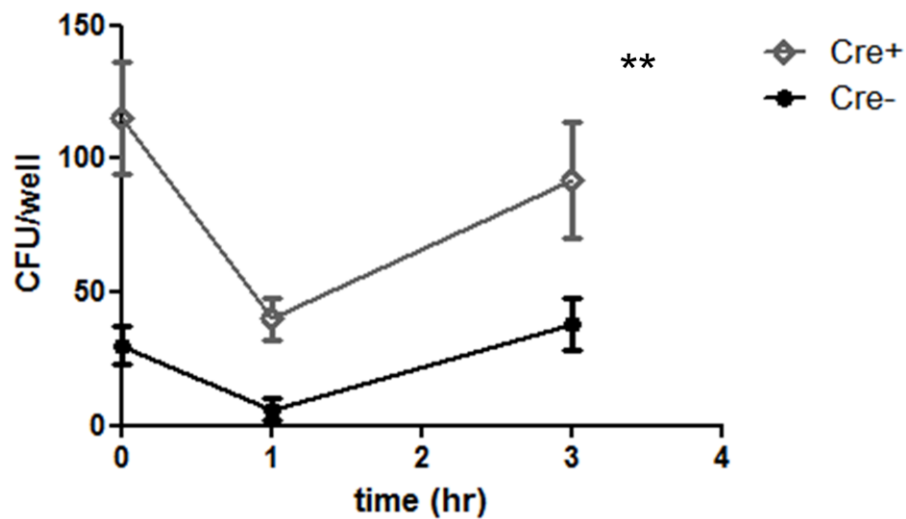


Figure 25: In-vitro macrophage infection with *Listeria monocytogenes* (MOI=1). Three-fold higher uptake of *Lm* in *LysMCre+* versus control macrophages is shown at time 0. A higher amount of *Lm* CFU is found inside *LysMCre+* cells compared to the *LysMCre-*. (** p<0.01)

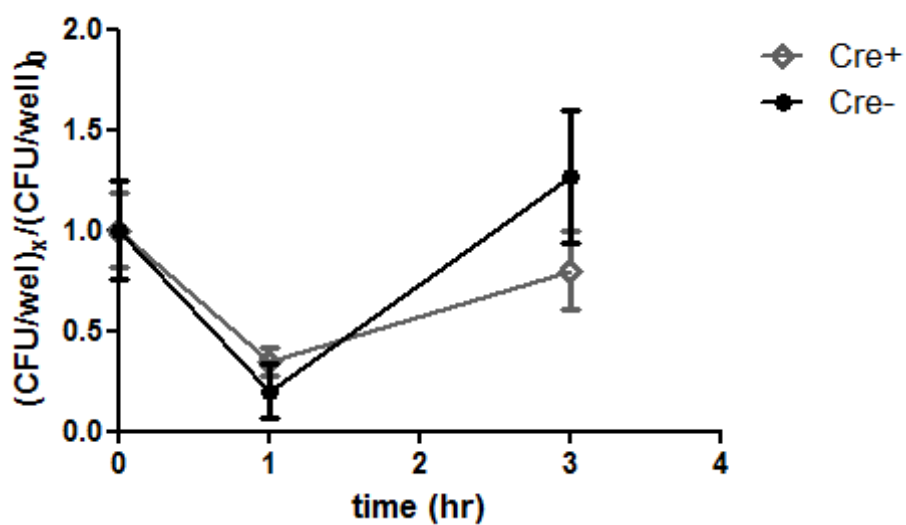


Figure 26: Normalized CFU data (MOI=1). *LysMCre+* and *LysMCre-* macrophages show the same efficiency in challenging *Lm* infections

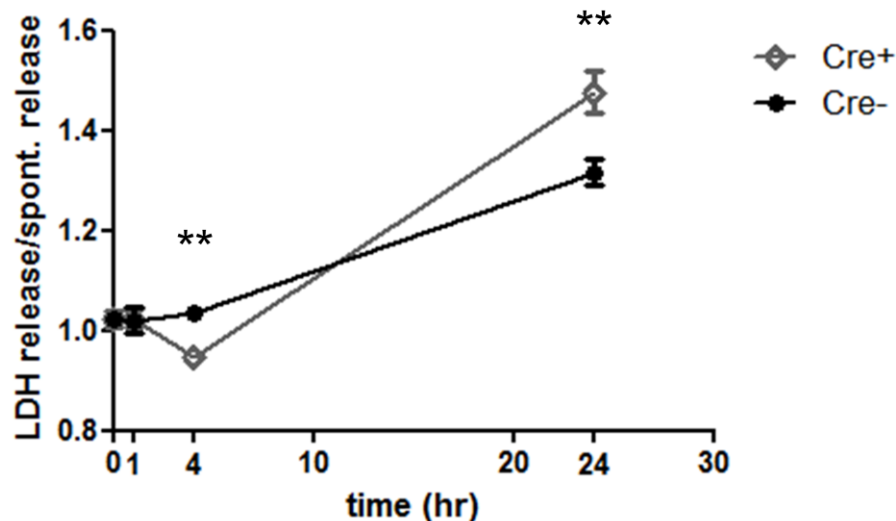


Figure 27: Cytotoxic effect of *Lm* in-vitro infection on macrophages. LDH release in *LysMCre+* infected macrophages is significantly higher than in the wild-type. (** $p < 0.01$)

Taken together, these data showed a significant difference between *LysMCre+* and *LysMCre-* macrophages in challenging *Listeria monocytogenes* infections: MnSOD knock-out macrophages were up-taking higher amounts of bacteria, which caused a higher cytotoxic damage and impaired bacterial clearance.

5.4.6 Conditional MnSOD deficient mice produce higher levels of MCP-1 in response to *Listeria monocytogenes* infection

Three days after intravenous infection with 10^5 CFU of *L. monocytogenes*, serum samples were analyzed for cytokine and chemokine production with a Luminex kit. Surprisingly, no difference was found in pro-inflammatory cytokine levels between *LysMCre+* and control mice. However, a three-fold higher concentration of MCP-1 was found in sera samples of *LysMCre+* mice (cf. Figure 28).

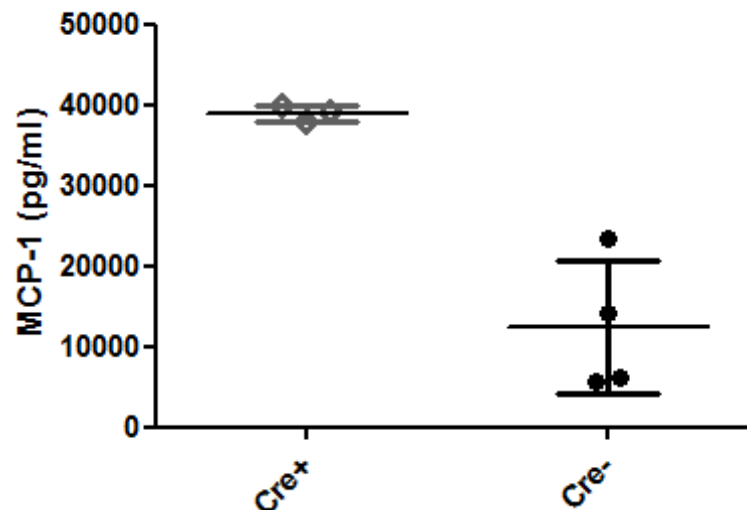


Figure 28: Monocyte chemoattractant protein-1 (MCP-1) concentration in serum samples at day three post-infection. In response to the *Listeria monocytogenes* infection, higher amounts of MCP-1 are produced and released in the bloodstream of *LysMCre+* mice than in controls. (**p<0.01)

5.4.7 MnSOD does not influence the response to *Listeria monocytogenes* in the intraperitoneal infection model

In order to investigate MnSOD role during *Lm* infection in the peritoneal cavity, *LysMCre+* and *LySMCre-* mice were infected intraperitoneally and the PECs recollected one hour post-infection. Collected cells were stained with F4/80-FITC, CD11b-APC, AnnexinV-PE and Propidium Iodide (PI). After FACS acquisition, macrophages were discriminated by the presence of the surface marker F4/80 or CD11b. The amount of dead cells was evaluated via Propidium Iodide (PI) and AnnexinV staining. Only cells which resulted F4/80 positive, AnnexinV negative and PI negative were counted as living macrophages (cf. Figure 29). All collected data were analyzed in terms of dead versus surviving macrophages. No significant difference was found between *LysMCre+* and *LysMCre-* mice in this infection model.

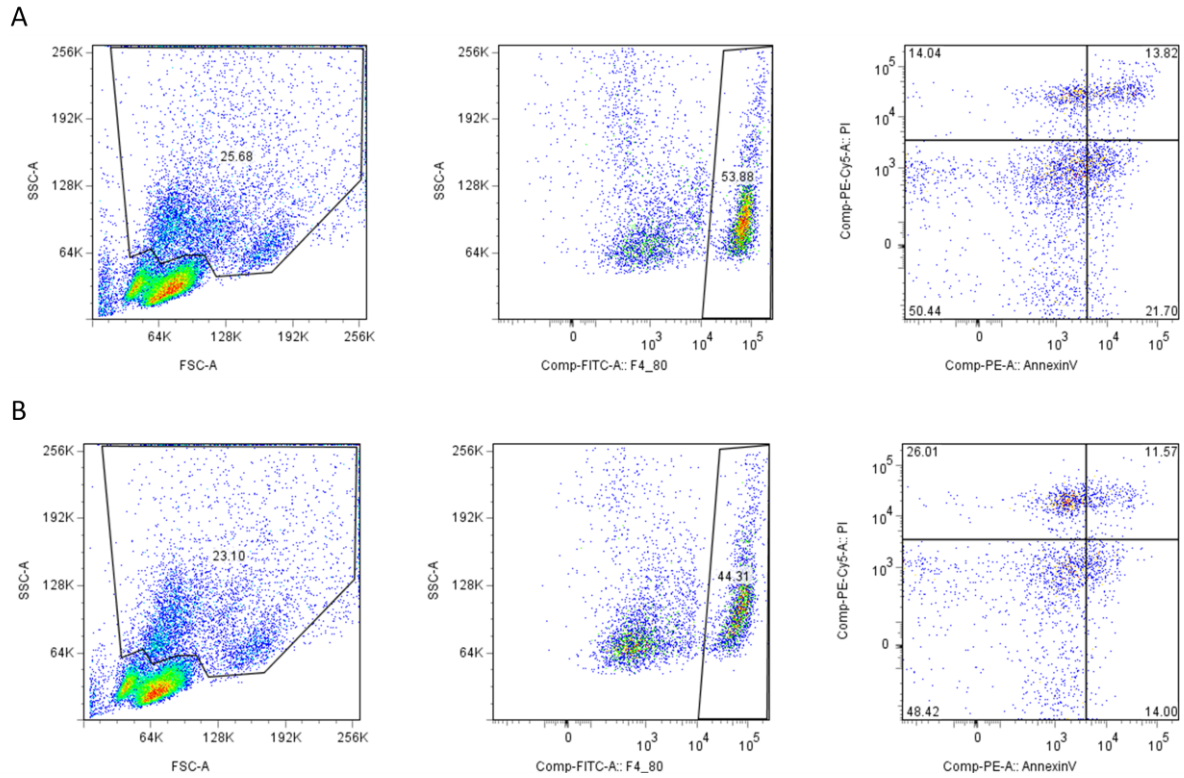


Figure 29: FACS analysis of PECs in mice infected intraperitoneally with *Lm*. Examples of PECS analyzed via FACS from *LysMCre*⁺ (A) and *LysMCre*⁻ (B) mice. The first gate (left) was set in order to include only cells in the analysis and get rid of cell debris. The second gate (centre) allows discrimination of macrophages (F4/80 pos) from other cell-types. On the right, macrophages analysis is shown.

6. Discussion of PART II

The aim of the second part of the present thesis was to investigate the involvement of MnSOD in host protection during infections. For this purpose, macrophage and/or neutrophil specific MnSOD knock-out mice were used for *in-vivo* infection models. Moreover, macrophages isolated from MnSOD conditional mutant mice were used for *in-vitro* experiments. Three different pathogens were used for the infections: *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Listeria monocytogenes*. All of these bacteria are Gram+. *Streptococcus pyogenes* and *Staphylococcus aureus* are extracellular pathogens, while *Listeria monocytogenes* is intracellular.

6.1 The *MnSOD^{fl/fl}LysMCre* line is suitable for the generation of macrophage specific MnSOD knock-out mice

Prior to infection experiments, efficiency and specificity of *LysMCre* deletion were evaluated. To this purpose, DNA was extracted from bone marrow derived macrophages (BMDM), T-cells, and B-cells isolated from *MnSOD^{fl/fl}LysMCre+* (*LysMCre+*) and *MnSOD^{fl/fl}LysMCre-* (*LysMCre-*), and the deletion assessed by Southern Blot analysis (see sections 2.11 and 2.12). The deletion proved to be efficient only in macrophages of the *LysMCre+* line (see section 5.1). Due to technical problems, it was not possible to show the presence of the “floxed” band in Southern Blot analysis of B-cells’ and T-cells’ DNA. B-cells and T-cells were isolated via cell-sorting, while macrophages were isolated and differentiated from the bone marrow. Southern Blot analysis was performed in two different experiments (one for BMDM, one for B- and T- cells). The conditions of DNA extraction, enzymatic digestion, electrophoretic run, blotting, and probe hybridization were the same in the two experiments. However, bands were present only in Southern blots of BMDM’s DNA. Hence, the substantial failure in showing the absence of the deletion in B- and T- cells is very likely due to the quality of the DNA. Due

to the lack of time, the experiment could not be further repeated. Nevertheless, the specific activation of the Lys-M promoter for macrophages and neutrophils has been already demonstrated [145, 152]. In the case of MnSOD gene, an ubiquitous inactivation can be excluded because the lethal phenotype resulting from the gene loss. For these reasons, the conditional knock-out model can be considered a valid instrument to investigate gene inactivation in macrophages and neutrophils.

6.2 MnSOD does not play a major role in host defense against *Streptococcus pyogenes* KTL-3 infection

Despite previous observation of MnSOD up-regulation during in-vitro infection of macrophages with *Streptococcus pyogenes* [89], no significant difference between MnSOD conditional knock-out mice and wild-type mice was found with respect to survival rates and bacteremia levels. Collected data showed an extremely high variance in both groups, with bacterial concentration in the blood ranging from 0 to 10^6 CFU/ml. The same variance in susceptibility to the infection was observed during survival rate experiments, where mice from the same group were successfully challenging the infection and eventually surviving, while other mice were dying by day two post infection. As it has been previously mentioned, all mice used during the experiments were not on a pure genetic background (see section 2.8). When the experiments started to be performed the *MnSOD^{fl/fl}LysMCre* breeding was supposed to be on a pure C57BL/6 genetic background. Nevertheless, after thorough examination of the back-cross breeding schemes, it was found that the number of backcrossing was not sufficient to guarantee a pure genetic background. Therefore, all mice of the above mentioned breeding were on a mixed genetic background, a random mosaic of SW129 and BL6 genes. SW129 strain is very susceptible to Streptococcal infections, whereas C57BL/6 has an intermediate resistance compared to other inbred mouse strains [153]. The observed phenotype seems to be

due to host genetic factors, which may possibly overcome MnSOD effect. Therefore, MnSOD involvement in host protection against *Streptococcus pyogenes* cannot be excluded, however it does not seem to play a major role. The MnSOD^{fl/fl} line is currently under speed-congenics backcrossing, and the described experiments will be repeated on conditional MnSOD knock-out mice on a pure C57BL/6 background as soon as the backcrossing will be ready.

6.3 MnSOD expression does not affect susceptibility to *Staphylococcus aureus* infection

Similarly to *Streptococcus pyogenes*, *Staphylococcus aureus* is an intracellular Gram+ bacterium. However, a mixed genetic background like the C57BL/6 - SW129 of the MnSOD^{fl/fl} line was not believed to be influent on the *S. aureus* infection model because BL/6 and SW129 mice were shown to have similar susceptibility to this pathogen [144]. In fact, both survival rates and bacteremia levels were much more similar within the experimental groups when compared to the outcome of streptococcal infections (see section 5.3). This infection model seems to be more solid in investigating MnSOD involvement during extracellular Gram+ bacterial challenge. Yet, *LysMCre*+ mice showed the same susceptibility displayed by *LysMCre*- mice, as inferred by the similar survival rates and bacterial burden in target organs such as liver and kidney.

6.4 MnSOD deficiency in macrophages and/or neutrophils increases susceptibility to *Listeria monocytogenes* infection

Survival rate of MnSOD conditional knock-out mice was significantly lower than wild-type mice (see section 5.4.2). Consistently with previous findings, about 70% of the wild-type animals were able to challenge the infections [99]. However, all the mice carrying the macrophage and/or neutrophil specific MnSOD gene mutation died within eight days after the

infection, indicating a major role of MnSOD expression as a host protective factor. In the *Lm* intravenous infection model, bacteria rapidly disseminate in liver and spleen of the animal. The immune reaction is mainly carried on by resident macrophages and the attracted neutrophils [122]. In the early phase of infection, cells of the innate immune system are responsible for controlling the infection and activating the adaptive immunity [122, 154]. A defective early response results in the presence of higher amounts of bacteria in these organs [155]. Similarly, in the present study high levels of *Lm* colonies were found in liver and spleen of infected animals 48 hours after the infection. However, the bacterial burden was significantly higher in *LysMCre*⁺ mice, thus suggesting that the incapability of controlling the infection in these organs might eventually lead to the observed lethal phenotype (see section 5.4.3). Tissue damage was confirmed by histopathological examination of liver and spleen sections. The histopathological analysis of liver and spleen slides in this experiment was consistent with previous studies employing the EGD strain of *Lm* for intravenous infections [156]. Such damage was significantly higher in *LysMCre*⁺ mice (see section 5.4.4). The observed lesions were larger and more severe in liver and spleen from MnSOD conditional knock-out, hence suggesting that organ failure is the cause of death. The link between uncontrolled bacterial growth and macrophage impaired functioning was provided by the *in-vitro* experiments (see section 5.4.5). MnSOD knock-out macrophages were up-taking significantly higher amounts of bacteria, resulting in a decreased killing ability and in a higher cytotoxic effect. Moreover, cytokine and chemokine analysis in serum samples of infected animals showed the presence of significantly higher levels of monocyte chemoattractant protein -1 (MCP-1) in conditional knock-out mice than in wild-type animals. Higher production of MCP-1 in response to *Lm* infection by susceptible strains has been shown in several publications [155, 156]. In the described context, the higher production of MCP-1 could be interpreted as the attempt to recruit more monocytes to the site of infection because of a higher damage.

In conclusion, MnSOD expression protects macrophages in the context of *Listeria monocytogenes* infection. MnSOD deficiency decreases liver and spleen resident macrophages defensive functions. This leads to the observed tissue damage and eventually to death.

6.5 Conclusion

MnSOD is a mitochondrial antioxidant enzyme. Its function has been investigated predominantly with regard to aging, cardiovascular diseases, neurodegenerative disorders, drug toxicity, and cancer [157-161]. Most of these studies were performed on heterozygous Sod2^{+/-} mice [162, 163]. To date, no involvement of MnSOD expression during infection has been reported.

In the present study, the macrophage and/or neutrophil specific MnSOD knock-out line was analyzed with respect to the phenotype during infection. A novel protective role of MnSOD during bacterial infection was found. Notwithstanding its mitochondrial location, MnSOD deficiency led to an increased susceptibility to the intracellular Gram⁺ pathogen *Listeria monocytogenes*. Previous observations of MnSOD up-regulation in macrophages infected with *Streptococcus pyogenes* [89] led to the hypothesis of this study that MnSOD over-expression might have a protective role during infection. While the findings concerning streptococcal infections cannot be interpreted due to the mixed genetic background (see section 6.3), the results obtained from an infection model with *Staphylococcus aureus*, another extracellular pathogen, did not support the hypothesized protective function of MnSOD. However, the fact that MnSOD was found to play a major role in host protection during *Listeria monocytogenes* suggests that the relevance of MnSOD may be specific for certain pathogens only. Although the findings from this study are not generalizable to any intra- or extracellular bacteria, they yield a preliminary insight into MnSOD protective

mechanisms. Considering that *Listeria monocytogenes* replication takes place inside macrophages, among other cell types [164], and the phagocyte-pathogen interaction is characterized by slower kinetics compared to staphylococcal challenge, it can be argued that the presence of MnSOD becomes more relevant during prolonged infection processes. In future research, it will certainly be interesting to investigate MnSOD involvement during parasitic infection.

Finally, an improved understanding of MnSOD function during infections could be helpful for developing more efficient therapeutic strategies aimed at enhancing innate host defense mechanisms by fostering anti-oxidant activity, rather than targeting the pathogen alone.

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Summary

This thesis comprises two parts. The first part concerns the generation of an IL-12p40 conditional knock-out mouse. ET cloning was employed to modify the endogenous locus of the *IL-12p40* murine gene contained in a BAC vector. The resulting targeting construct was capable of recombining homologously within ES cells, which were subsequently injected into blastocysts in order to generate a conditional knock-out mouse line. None of the generated chimeras could be tested for germ-line transmission due to early lethality and sterility.

In part two, macrophage and/or neutrophil specific MnSOD knock-out mice were obtained through the breeding of MnSOD^{fl/fl} to LysM-Cre mice. Initially, the LysMCre-driven deletion of the loxP-flanked exon3 of MnSOD was shown to be specific and efficient. Subsequently, three different infection models, namely *Streptococcus pyogenes*, *Staphylococcus aureus* and *Listeria monocytogenes*, were tested in order to investigate the role of MnSOD during both extra-cellular and intra-cellular bacterial infections. The MnSOD^{fl/fl} x LysMCre breeding was not on a pure C57BL/6 genetic background. Thus, the results concerning the streptococcal infection model are not interpretable at this point. MnSOD deficiency was found to be irrelevant for the outcome of the infection during *Staphylococcus aureus* intravenous infections. However, a major protective role of MnSOD in Listeriosis models was established. Macrophage and/or neutrophil specific MnSOD knock-out mice displayed a significantly higher susceptibility to intravenous *Listeria monocytogenes* infection compared to controls, as implied by a lower survival rate and higher bacterial burden in liver and spleen. Moreover, *in-vitro* experiments showed a higher bacterial up-take and a lower efficiency in killing bacteria.

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